



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/56, 15/85, C12Q 1/68	A1	(11) International Publication Number: WO 98/49320 (43) International Publication Date: 5 November 1998 (05.11.98)
(21) International Application Number: PCT/CA98/00157 (22) International Filing Date: 26 February 1998 (26.02.98) (30) Priority Data: 2,203,613 24 April 1997 (24.04.97) CA (71) Applicants (for all designated States except US): HER MAJESTY IN RIGHT OF CANADA, as represented by THE MINISTER OF AGRICULTURE AND AGRI-FOOD CANADA Central Experimental Farm [CA/CA]; Ottawa, Ontario K1A 0C6 (CA). UNIVERSITY OF OTTAWA [CA/CA]; Research Services, 550 Cumberland, Ottawa, Ontario K1N 6N5 (CA). (72) Inventors; and (75) Inventors/Applicants (for US only): BENKEL, Bernhard, F. [CA/CA]; 293 Summit Avenue, Ottawa, Ontario K1H 5Z7 (CA). NGUYEN, Thuy, H. [CA/CA]; Apartment 1201, 1316 Carling Avenue, Ottawa, Ontario K1Z 7L1 (CA). AHLUWALIA, Navneet, K. [CA/CA]; Apartment 901, 1316 Carling Avenue, Ottawa, Ontario K1Z 7L1 (CA). FALCONER, Marcia, M. [CA/CA]; 33 Abingdon Drive, Nepean, Ontario K2H 7M5 (CA). GAVORA, Jan, S. [CA/CA]; 51 Forest Hill Avenue, Ottawa, Ontario K2C 1P7 (CA). HICKEY, Donal, A. [CA/CA]; 36 Burnham		Road, Ottawa, Ontario K1S 0J8 (CA). BENKEL, Kaarina, I. [CA/CA]; 293 Summit Avenue, Ottawa, Ontario K1H 5Z7 (CA). (74) Agents: ERRATT, Judy et al.; Gowling, Strathy & Henderson, Suite 2600, 160 Elgin Street, Ottawa, Ontario K1P 1C3 (CA). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>

(54) Title: SECRETED α -AMYLASE AS A REPORTER GENE**(57) Abstract**

The regulation of gene expression is of fundamental importance to all biological functions including adaptation to environmental conditions, cell division and differentiation, and the development of disease states such as cancer. The application of "reporter" gene systems, which allow changes in gene expression to be assayed quickly and easily, has contributed greatly to our understanding of the mechanisms involved in gene regulation. A reporter gene system comprising a gene encoding an α -amylase enzyme for use in transforming mammalian cells is described. In a further example the reporter gene system comprises a second α -amylase gene, which codes for an enzyme with an electrophoretically distinct mobility from that of the first α -amylase. The two genes are used for simultaneous expression in mammalian cells, with one isozyme acting as an internal control, and the other isozyme acting as the indicator gene. The use of α -amylase as a secreted reporter gene is particularly attractive because the enzyme is extremely stable over a wide range of conditions. The measurement of α -amylase activity is simple, quantitative, sensitive, safe and inexpensive, and the range of electrophoretically distinguishable variants available allows the assays to be performed in virtually any host without interference from endogenous amylase activities.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

SECRETED α -AMYLASE AS A REPORTER GENE

The present invention relates to a secreted reporter gene system. More specifically, the present invention relates to a secreted reporter gene system based on one or more α -amylases, which are electrophoretically distinct variants of each other, for simultaneous expression in mammalian cells.

BACKGROUND OF THE INVENTION

Reporter genes come in two basic varieties; those that encode intracellular enzymes, and those that code for secreted reporter proteins. Most reporter genes currently in use are of the intracellular variety that require manipulation of the cells, normally harvesting and lysis, prior to measurement of the reporter activity. In contrast, cells transformed with constructs that encode secreted reporter enzymes do not require manipulation or lysis of the cells for activity measurements. Instead, aliquots of the extracellular medium are harvested and assayed directly for enzyme activity. Thus, secreted reporter genes can be used to monitor gene expression *in vitro*, *in vivo* and *ex vivo*.

There are a number of commercially available reporter genes that encode intracellular enzymes, e.g. green fluorescent protein (GFP; Chalfie *et al.*, 1994, Science 263: 802-805), luciferase (de Wet *et al.*, 1987, Mol. Cell. Biol. 7: 725-737), chloramphenicol acetyltransferase (CAT; Gorman *et al.*, 1982, Mol. Cell. Biol. 2: 1044-1051). However, none of these intracellular enzymes are suitable as a secreted reporter activity. For instance, the luciferase reporter gene is used widely due mainly to the sensitivity of the detection system and the lack of background activity in most biological systems. However, the luciferase enzyme is relatively unstable under any conditions, and is completely and irreversibly inactivated by the process of secretion.

Secretable human placental alkaline phosphatase (SEAP); a truncated form of membrane-bound alkaline phosphatase; (Berger, J., Hauber, J., Hauber, R., Geiger, R., Cullen, B.R. 1988. Gene. 66: 1-10; Cullen, B.R., and Malim, M.H. 1992.

Methods Enzymol. 216: 362-368) is a reporter gene that is distributed by Clontech as a secreted reporter gene/enzyme system (see European Patent application 0 327 960). However, many biological systems display endogenous (background) alkaline phosphatase (AP) activity. For example, the high level of alkaline phosphatase activity in cows' milk precludes the use of SEAP as a reporter activity for experiments aimed at using the bovine udder as a "bioreactor" in the production of bioengineered milk. A second commercially-available, secreted reporter gene is human growth hormone (hGH, Boehringer Mannheim; Selden, R.F., Howie, K., Rowe, M.E., Goodman, H., Moore, D. 1986, Mol. Cell. Biol. 6: 3173-3179). However, since hGH has no easily measurable biological activity, the detection system for this secreted product uses a laborious, indirect antibody-based ELISA method.

Thus, there is a need for an improved secreted reporter gene that overcomes the problems of the prior art systems. Specifically there is a need for a secreted reporter gene encoding a biological activity that, unlike hGH, is safe and easily measured, but that does not have the background activity problems associated with SEAP.

SUMMARY OF THE INVENTION

Thus according to the present invention there is provided a secreted reporter gene system for mammalian cells. More specifically the present invention relates to a secreted reporter gene system based on one or more variants of α -amylase, that have different electrophoretic mobilities from that of mammalian α -amylases, and that can be efficiently expressed and secreted by mammalian cells.

The reporter gene system of the present invention comprises a DNA sequence with the following components: 1) a signal peptide coding region; 2) a sequence encoding one or more variants of the α -amylase mature protein; and 3) a transcription termination region.

In one embodiment of the present invention, the α -amylase gene is the chicken (*Gallus gallus*) α -amylase gene.

In one embodiment of the present invention, the α -amylase gene is the fruit fly (*Drosophila melanogaster*) α -amylase gene.

5 In one embodiment of the present invention, the reporter gene system comprises a DNA sequence based on the α -amylase gene from the chicken (*Gallus gallus*), in conjunction with the α -amylase gene from the fruit fly (*Drosophila melanogaster*), in a dual-reporter plasmid.

10 In a further embodiment of the present invention, the signal sequence is based on the *Drosophila melanogaster* α -amylase secretion signal.

In yet a further embodiment of the present invention the transcription termination region is based on the *Drosophila melanogaster* α -amylase gene termination region.

15 In a further aspect of the present invention the reporter gene system also includes a transcription promoter region.

The present invention is also directed to a vector comprising the reporter gene system.

20

The present invention also defines a procedure to assay for α -amylase activity in complex biological mixtures of proteins.

25 In yet a further embodiment of the present invention, there is provided a plate diffusion assay, which is used to measure α -amylase activity from a large number of samples.

Also included within the scope of the present invention is a kit including the reporter gene system of the present invention and reagents for testing α -amylase activity.

30

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

FIGURE 1 shows the sequence of the chicken α -amylase cDNA (SEQID No: 1).

5 **FIGURE 2** shows an example of the reporter gene construct which does not contain a promoter region. The α -amylase mature peptide coding sequence '*amylase*', and transcription termination region '*ter*' are indicated. The solid black rectangle between the promoter and the amylase coding region represents the secretion signal peptide. Restriction enzyme recognition sites are as follows:
10 *As* = *AscI* ; *Hi* = *HindIII*; *Nc* = *NcoI*; *Nh* = *NheI*; *No* = *NotI*. The 8-cutter sites *NotI* and *AscI* were built into the upstream and downstream ends, respectively, of the reporter gene in order to facilitate its insertion into the reporter construct.

FIGURE 3 shows an example of the reporter gene construct which contains a promoter region. The description is as above for Figure 2, except the
15 transcription promoter '*pro*' is also indicated.

FIGURE 4 shows the sequence of the transcription termination region (SEQID No: 2).

FIGURE 5 shows the electrophoretic mobilities of α -amylases from 3 different sources in a native gel assay. Lane 1, pig pancreatic amylase; Lane 2, *D.*
20 *melanogaster* amylase; Lane 3, chicken pancreatic amylase.

FIGURE 6 shows the production of the reporter activity by mammalian cells in culture. Lane 1, chicken pancreatic amylase control; Lane 2, mouse (PG13) cells; Lane 3, mouse (PA317) cells; Lane 4, hamster (CHO) cells; Lane 5, monkey (MA104) cells; Lane 6, cow (ET2) cells; Lane 7, human (Hela) cells;
25 Lane 8, untransformed mouse cells (PG13).

FIGURE 7 shows the expression levels of amylase under different promoters. Lane 1, medium from control, untransformed PA317 cells; Lane 2, cells transformed with reporter construct containing *LTR:amy* reporter gene; Lane 3, cells transformed with reporter construct containing *Actin:amy* reporter gene; Lane
30 4, cells transformed with reporter construct containing *CMV:amy* reporter gene; Lane 5, chicken amylase standard.

FIGURE 8 shows the detection of amylase activity in cows' milk. Lane 1, whole milk; Lane 2, whole milk spiked with chicken amylase; Lane 3, empty; Lane 4, chicken amylase standard; Lane 5 & 6, milk following removal of caseins; Lanes 7 & 8, milk with amylase spike following removal of caseins.

5 **FIGURE 9** shows the isolation and detection of α -amylase in cows' pre-lactation secretions (pre-milk). Lanes 1, 2 & 3, early premilk from 3 quarters of cow #99. Lane 1, control quarter (RF-); Lane 2, treated quarter (RH+); Lane 3, treated quarter (LH+). Lanes 4, 5 & 6, late premilk from cow #99. Lane 4, treated quarter (LH+), Lane 5, treated quarter (RH+); Lane 6, control quarter (RF-). Lane 7, mixture of pig and chicken amylase standards; Lane 8, chicken amylase standard.

FIGURE 10 is a schematic depiction of the plasmids used in the dual-reporter system.

15 "Fly" refers to *D. melanogaster*; "chick" refers to *G. gallus*. Abbreviations are: ss=signal sequence; amy= α -amylase; CMVp=immediate early promoter of the human cytomegalovirus; LpA=late polyadenylation site; ori=origin of replication; TB=transcriptional blocker; actin p=rat β -actin promoter.

20 **FIGURE 11** shows the production of α -amylase reporter activities by cultured mammalian cells transfected with either the pUC-TEST plasmid or the pUC-CONTROL plasmid. Lane 1, chicken amylase standard; Lane 2, fly amylase standard; Lane 3, medium from control, untransformed PA317 cells, Lane 4, cells transformed with pUC-TEST=base dual reporter construct; Lane 5, cells transformed with pUC-CONTROL=dual reporter construct containing the actin promoter inserted upstream of the fly amylase reporter gene.

25 **FIGURE 12** shows the reporter activities of PA317 cells co-transformed with chicken α -amylase and the firefly luciferase. The reporter genes in each co-transformation were placed under the same promoter: the murine retroviral LTR, the rat β -actin promoter, or the human CMV early immediate promoter. Results for amylase activity were obtained by densitometric scanning of the photograph of the native gel containing the amylase activity obtained from the cell supernatant. Luciferase activity was assayed from cell lysates according to manufacturer's instructions (Promega Corp).

30

DESCRIPTION OF PREFERRED EMBODIMENT

The present invention relates to a secreted reporter gene system. More specifically, the present invention relates to a secreted reporter gene system based on one or more α -amylases, which are electrophoretically distinct variants of each other, and also distinct from mammalian α -amylases, for simultaneous expression in mammalian cells.

An α -amylase reporter gene encodes a biological activity that is easily measured by a variety of liquid or semi-solid phase assay systems. The use of a native gel assay system eliminates any potential interference by endogenous amylase activities produced by the transformed, mammalian target cells. In addition, the advantage of using a secreted reporter activity is that it is not necessary to harvest cells from the culture dish, or to biopsy a transformed tissue (e.g. udder epithelium), nor is it necessary to lyse cells prior to assay. Instead, the cell culture supernatant or biological fluid (e.g. milk) is recovered from the transformed culture or animal and assayed for reporter activity. This feature has several advantages such as the ability to conduct multiple measurements over time on a single transformed population of cells (time course measurements).

Since α -amylase exists in a number of distinct isoforms, an additional advantage to using the amylase reporter system is that a different isozyme can be used as an internal control in transfection experiments. The "normalizing" isozyme reflects a standard level of gene expression, while the "test" isozyme is the indicator enzyme. Since the isozymes have different migration patterns on the native gel, they can be assayed simultaneously within the same gel lane. This simplifies the assay procedure, in contrast to other reporter systems where the test and normalizing activities require separate assays.

The chicken and *Drosophila* α -amylase enzymes are two examples of suitable α -amylases, which can be used alone or together in both embodiments of the present invention. These enzymes are compatible with the mammalian cellular translation and

secretion machinery. Therefore, the mammalian cells are able to produce and secrete them efficiently in an authentic, bioactive form. Furthermore, these α -amylases have the same physio-chemical requirements for optimal activity and stability, for example, pH optimum, calcium activation, etc., as mammalian amylases. These features are essential for their use as reporter activities. In addition, the electrophoretic mobilities of the mature, active chicken and *Drosophila* α -amylase enzymes on native polyacrylamide gels are different from that of mammalian amylases. Therefore if any amylase enzyme is secreted from mammalian cells, the reporter activities can easily be differentiated from the background activity.

The absence of background activity is particularly important when measuring reporter activities in biological fluids, such as cows' milk, which contains high background levels for other reporter systems, e.g. the SEAP reporter system. According to the present invention, the α -amylase reporter system is most useful in complex protein mixtures such as biological fluids, specifically fetal calf serum, milk, and pre-lactation secretions, because the reporter α -amylase activity can be quantitatively recovered, using the isolation procedure included in this invention, from such fluids, which will be described in detail below.

Included within the scope of the present invention are modified DNA sequences encoding functionally active α -amylases from chicken and *Drosophila*. Possible modifications include but are not limited to: 1) nucleotide substitutions that eliminate restriction enzyme (RE) sites in the naturally-occurring sequence, but do not alter the amino acid sequences, and the use of these RE sites in a multiple-cloning-region for the introduction of DNA components, e.g. the transcription promoter; 2) conservative amino acid substitutions; 3) the enhancement of the specific activity of the reporter polypeptide by amino acid substitutions; 4) increasing the temperature stability of the reporter enzyme by amino acid substitutions; 5) creation of hybrid reporter enzymes from portions of genes coding for different isozymes. Also included is the potential use of amylase inhibitors to specifically inhibit any endogenous, mammalian amylases that might be present in the extracellular medium of the transformed cells.

The reporter gene system of the present invention also includes a DNA sequence encoding a signal peptide coding region, which in one embodiment is based on the *Drosophila melanogaster* α -amylase pre-protein. This signal peptide works effectively in combination with the chicken α -amylase enzyme and the mammalian cellular secretion machinery. The DNA sequence encoding the signal peptide was modified to include useful restriction enzyme sites for the introduction of promoter sequences to drive the reporter gene. The present invention also encompasses any modifications of the signal peptide region that may increase the efficiency of the secretion process beyond the current level.

The reporter gene system of the present invention also includes a transcription termination region. In one embodiment of the present invention the transcription terminator region is derived from the *Drosophila melanogaster* amylase gene. This portion of the reporter gene serves the following functions: (1) to provide a 3' non-coding region for the transcript; (2) to provide a transcription termination signal for the RNA polymerase complex; and (3) to provide a poly-adenylation motif to the primary transcript. In the example where two amylase genes are used in the reporter gene system and one of said genes is the *D. melanogaster* α -amylase gene, the terminator region comes from the late polyadenylation site of SV40, to minimize possible recombination with the α -amylase cassette.

Included within the scope of the present invention is the addition of any functional DNA elements, such as transcription enhancer elements, introns, etc., to the transcription terminator region in order to enhance the overall level of expression of the reporter gene.

In one embodiment of the present invention, the reporter gene system also includes a DNA sequence encoding a transcription promoter region. When the reporter gene system of the present invention includes a promoter, the primary use of this secreted reporter gene system is to monitor the efficiency of the introduction of foreign DNA into mammalian cells *in vivo*, *in vitro* and *ex vivo*. This application

facilitates experiments designed to optimize the conditions, methods and vehicles employed for the introduction of foreign DNA into mammalian cells. There are a number of high expression level, commercially available promoters which are suitable for this application. Not to be construed as limiting, possible examples include: a
5 Cytomegalovirus promoter, a murine retroviral promoter and a rat beta-actin promoter.

One aspect of the invention would be the use of a tissue-specific promoter to drive the expression of the reporter gene. This configuration would enable the researcher to distinguish between expression in target and non-target cell populations.
10 Furthermore, it would allow researchers to optimize their delivery systems for targeting the foreign DNA to cell types that have the greatest capacity for producing and secreting foreign peptides. This aspect is especially important for *in vivo* gene transfer. Specific applications would include gene transfer to udder epithelial cells in mammals for the production of pharmaceutical agents into the milk (the somatic
15 bioreactor concept), and DNA transfer for gene therapy in humans.

The construction of the reporter gene involves the use of standard recombinant DNA methods, including the use of restriction enzymes to fuse DNA molecules with coherent ends, the amplification of DNA fragments using the polymerase chain reaction
20 (PCR; Canadian patent 1,237,685), and the synthesis of DNA fragments using phosphoramidite chemistry (Matteucci and Caruthers, 1981, J. Am. Chem. Soc. 103: 3185-3191). The application of the reporter gene involves the insertion of the gene into a suitable vector to form double-stranded, circular DNA molecules that are delivered to the eukaryote cells. General methods for the preparation and modification
25 of recombinant DNA molecules have been described by Cohen *et al.* [U.S. patent No. 4,237,224], Collins *et al.* [U.S. patent No. 4,304,863], Sambrook *et al.* (1989, *Molecular cloning: A laboratory manual*, Cold Spring Harbor Laboratory), and Mullis and Faloona (1987, *Methods Enzymol.* 155: 335-350). Because they illustrate much of the state of the art, these references are hereby incorporated by reference.

Once assembled, the vector carrying the reporter gene is delivered to the target cells by one of a number of commonly used methods, e.g. viral vectors, calcium phosphate co-precipitation, or liposome-mediated delivery. The DNA plasmid vector used to carry the reporter gene can provide for either transient or stable maintenance of the reporter gene in the target, host cells depending upon the type of vector employed.

The production and secretion of proteins, including reporter enzymes, by eukaryotic cells is a multi-stage process. Briefly, the process begins with the transcription of the gene encoding the secreted product, followed by the processing of the primary transcript into mRNA, and the transport of the mature messenger into the cytoplasm. The mRNA is translated into the preprotein and simultaneously translocated into the first compartment of the secretory pathway by ribosomes associated with the endoplasmic reticulum. Targeting of the preprotein to the secretion pathway is mediated by a secretion signal peptide. Within the endoplasmic reticulum, golgi apparatus and the secretory vesicles, the primary translation product undergoes sorting and maturation to produce the final bioactive protein which is released into the extracellular medium. The present invention provides for novel reporter activities based on the chicken α -amylase gene and other electrophoretically distinct variants of α -amylase, which are compatible with and can be secreted efficiently by mammalian cells. The present invention also includes a secretion signal sequence that effectively targets the reporter peptide to the secretory machinery of mammalian producer cells.

There are a number of prior reports on the expression and secretion of α -amylase in microbial systems. In addition, Japanese Abstract 63263086 described the use of α -amylase as an indicator gene in a "promoter trap" construct for use in both prokaryote and eukaryote systems. The present system however is preferred, in that this system is designed to measure gene expression and DNA delivery specifically in mammalian cells using electrophoretically distinguishable variants in combination with an assay system optimized for use with biological fluids.

The present invention also includes an isolation procedure whereby the reporter activity can be quantitatively recovered from complex protein mixtures such as biological fluids, specifically fetal calf serum, milk, and pre-lactation secretions. The isolation protocol, though based on the procedures of Vretblad, P., 1974, FEBS Lett. 47(1): 86-89, Silvanovich, M. P., & Hill, A. D., 1976, Anal. Biochem. 73: 430-433, and Iefuji, H., et al., 1996, Biochem. J. 318: 989-996, was adapted especially for use in concentrated protein mixtures. Due to the high protein content, the reporter activity cannot be assayed using the original protocols. The present protocol differs from the original procedures in that 1) the non-specific proteins in the fluid are removed by addition of 40% ethanol, and 2) the ethanol-containing solution is applied directly onto the immobilized β -cyclodextrin to further reduce the nonspecific background.

The present invention also includes a "plate diffusion" assay which is one method for measuring the reporter activity when large numbers of samples, for example, culture supernatants from a population of stably selected clones, must be assayed. The method described here is adapted from the observation that colonies of amylase-producing microorganisms form clearing-zones on starch plates (Iefuji, H., et al. 1994. Biosci. Biotechnol. Biochem. 58: 2261-2262). In the amylase reporter system, the amylase-containing solutions are applied to wells formed in starch-containing agarose plates; diffusion of the solution through the starch-agarose forms clearing zones whose size correlates with the concentration of amylase in the solution.

Under conditions where cells are transiently transfected with the reporter plasmid, the amount of secreted reporter activity measured in the extracellular medium of a population of cells, transformed with a multicopy plasmid carrying a reporter gene which is driven by a strong, systemic promoter, is determined by two variables: 1) the number of cells that have received the reporter construct and; 2) the average number of copies of the DNA construct that each transformed cell received. Thus, under these conditions, the secreted reporter activity provides a measure of the efficiency of the DNA delivery system, i.e. how many cells in the population were transformed with

foreign DNA and how many copies, on average, of the transforming vector were incorporated into each transformed cell.

Under conditions where the cells are stably transfected with the reporter plasmid, the level of secreted reporter activity would, in addition, depend on: 1) the site at which the reporter gene becomes integrated, 2) the metabolic state of the cells, and 3) the environmental conditions that may regulate the cell's metabolism in general, and the promoter that drives the reporter gene in particular. If two of the above conditions are experimentally controlled, the secreted reporter gene can be used to monitor the effects of the third variable in time-course-type experiments.

The present invention also includes amylase reporter kits. Kits containing a single or double amylase gene are included within the scope of the present invention.

The single reporter kit would include: two DNA constructs; a plate assay means; and instructions for use. The single reporter kit would optionally include a beta-dextrin purification system, which would be needed in high-protein fluids to allow detection of the amylase activity.

The first DNA construct includes a test plasmid containing a single gene coding for a single amylase isozyme, with a multiple cloning region just upstream where a test promoter can be inserted. The second DNA construct includes a control plasmid where the gene is placed under control of a strong promoter. The plate assay detects amylase activity very simply. It consists of : a mould to form the gel plate; a buffer used to make the agarose media; detection solutions; and a sample of amylase as a positive control. The beta-dextrin system, if included, removes non-specific proteins from high-protein fluids to allow detection of the amylase activity. It consists of: an immobilized beta-dextrin packed into columns; equilibration buffer; wash buffer; and elution buffer. The buffers could be included within the kit or be substituted with instructions on the buffer preparation. The kit will also include a protocol booklet, which would give detailed procedures, as well as simple steps used

to remove background signals from high-protein biological fluids such as serum-containing media, pre-lactation secretion, and milk.

5 The dual amylase report kit will include: two DNA constructs; a gel electrophoresis assay means; and instructions for use. The dual reporter kit would optionally include a beta-dextrin purification system, which would be needed in high-protein fluids to allow detection of the amylase activity.

10 In this system the first DNA construct includes a test plasmid (containing the genes coding for both amylase isozymes, one of which is under a "normalizing" promoter). The second DNA construct includes a control plasmid containing the isozymes under different promoters. Gel electrophoresis allows unambiguous identification of the 2 isozymes. The assay consists of: a sample buffer; gel preparation buffer; detection solutions and amylase isozymes as positive controls. As
15 with the single amylase reporter kit the buffers can be included within the kit or they can be replaced with instructions on the buffer preparation.

If included, the beta-dextrin system is identical to that contained in the Single Reporter Kit and the protocol booklet is identical to that contained in the Single
20 Reporter Kit.

The kit of the present invention is much more inexpensive than any expression kit presently available. Furthermore, amylase is a very stable enzyme, whose signal improves with duration of expression. The dual expression kit allows for
25 normalization of the expression data. Endogenous levels, as mentioned previously are not a problem with this detection system.

While this invention is described in detail with particular reference to preferred embodiments thereof, said embodiments are offered to illustrate but do not limit the
30 invention.

EXAMPLES**EXAMPLE 1: Chicken α -amylase cDNA**

In order to isolate a chicken genomic α -amylase clone, a commercial chicken library in the vector λ FIX II (Stratagene) was screened with a murine amylase cDNA probe (1.2 kbp *Pst*I fragment of pMPa21, Hagenbuechle *et al.*, 1980, Cell 21: 179-187). Hybridization was carried out under conditions of reduced stringency at 37°C in a standard buffer system containing 50% formamide (Benkel and Gavora, 1993, Animal Genetics 24: 409-413). Following hybridization, the filters were washed twice for 15 min each in 2x SSC, 0.1% SDS at 42°C, followed by a single wash in 0.5x SSC, 0.1% SDS at 50°C for 30 min. Autoradiograms were exposed at -70°C with intensifying screens.

Eighteen positive signals were detected following the first round of library screening. Ten plaques were chosen at random for second screening, and 4 of these isolates were still positive after a third round of screening. One clone (λ A-1) was chosen for large-scale DNA isolation and fragment subcloning in preparation for sequence analysis.

A series of overlapping subclones spanning the amylase genomic region was constructed by inserting restriction fragments of the primary isolate λ A-1 into the vector pUC18. Double-stranded DNA was sequenced by the gene-walking method using synthetic oligonucleotide primers. Primers were synthesized on an Applied Biosystems 392 synthesizer and deblocked and desalted before use. Sequencing reactions were performed using the dye terminator cycle sequencing kit as described in the instructions supplied by the manufacturer (Applied Biosystems Inc.). The extension products were analyzed on an Applied Biosystems 373A automated sequencer, and sequence assembly was performed using MicroGenie software by Beckman.

The chicken amylase coding region was prepared using reverse transcription-polymerase chain reaction technology (RT-PCR). Oligonucleotide primers for the PCR

step were designed based on the chicken genomic amylase sequence derived as described above. Approximately 2 μ g of total RNA from chicken pancreas was used as substrate in a reverse transcription (RT) reaction using the Perkin Elmer RT-PCR kit components according to the instructions supplied by the manufacturer. The RT-reaction was primed with oligo-dT. The oligonucleotide primers used for the PCR stage of the RT-PCR reaction were as follows: (i) *Chamy-Nhe* (5'-ATGCTAGCTCAGTACAATCCCAACACTCAGGCT-3', SEQID No: 3) which spans the position in the coding region corresponding to the N-terminus of the mature enzyme; and (ii) *Chamy-Hin* (5'-CGAAGCTTATAACTTGGCATCA
ACGTGAATTG-3', SEQID No: 4) which spans the stop codon of the amylase coding region. The primers were designed to amplify the region in the gene encoding the mature α -amylase peptide. In addition, *Chamy-Nhe* converts the environment of the signal peptidase cleavage site into an *NheI* site, while *Chamy-Hin* adds a *HindIII* site immediately downstream of the stop codon (enzyme recognition sites are underlined). The modifications introduced by the PCR primers allow the amplified cDNA to be inserted into the reporter construct using cohesive overhang ligation.

PCR amplification of the chicken amylase cDNA was performed using the LA-PCR kit as described in the manufacturer's product bulletin (TaKaRa). The amplified fragment was sequenced using PCR-based chain termination technology, prior to insertion into the reporter construct - this sequence, SEQ ID No: 1, is shown in Figure 1.

EXAMPLE 2: Signal Peptide Coding Region

The signal peptide used in the current reporter gene is modelled on the sequence found in the *Amy1* gene of the Oregon-R strain of *Drosophila melanogaster* (Boer and Hickey, 1986, Nuc. Acids Res. 14: 8399-8411). The sequence of the native signal peptide extends from nucleotide 1 to 54 in Figure 2 of that reference. The 5'-end of the sequence encoding the signal peptide was modified to incorporate an *NcoI* site straddling the translation start codon (ATG) - i.e. the sequence AT ATG T was changed to CC ATG G. This modification changes the amino acid immediately downstream of the initiator

Met from a Phe to a Val. In addition, the 3'-end of the signal sequence was modified to accommodate an *NheI* site. Here, the original *D. melanogaster* sequence of --Ala-Asn-Ala-- (GCC AAC GCC) was changed to --Ala-Leu-Ala-- (GCG CTA GCC). The DNA fragment encoding the signal peptide was constructed in the laboratory on a DNA synthesizer, and assembled into the reporter construct using the restriction enzyme sites built into its ends. The novel signal peptide sequence used in the reporter construct has the following sequence (SEQID No: 5).

```

5 ' -CCATGGTTCTGGCCAAGAGCATAGTGTGCCTCGCCCTCCTGGCGGTGGCGCTAGCT- 3 '
10 3 ' -GGTACCAAGACCGGTTCTCGTATCACACGGAGCGGGAGGACCGCCACCGCGATCGA- 3 '
      NcoI                               NheI

```

15 EXAMPLE 3: Promoter Region

In this example the immediate early promoter of the Human cytomegalovirus (CMV; Stinski and Roehr, 1985, J. Virology 55: 431-441) was used to drive the expression of the reporter gene.

20 Other examples of promoters that have been used successfully in the reporter gene include; a murine retroviral LTR, which is a composite of the MMTV (Mouse Mammary Tumor Virus; Ponta *et al.* 1985, Proc. Natl. Acad. Sci. 82: 1020-1024) and the MoMSV (Moloney Murine Sarcoma Virus; Lin *et al.* 1990, Proc. Natl. Acad. Sci. 87: 36-40) LTRs, and the rat beta-actin promoter (Nudel *et al.* 1983, Nucl. Acids Res., 11, 25 1759-1771).

DNA fragments to be used as promoters were amplified by PCR using oligonucleotide primers that incorporated useful restriction enzyme sites at the upstream (*NotI*) and downstream (*NcoI*) ends of the amplified promoter sequences. Restricted promoters fragments were inserted into the reporter gene construct by cohesive end ligation.

EXAMPLE 4: Transcription Termination Region

The final component of the reporter gene is the transcription terminator. In this example 567 bp of the *Drosophila melanogaster* Oregon-R amylase gene termination region was used. The sequence of this section (SEQID No: 2) is shown in Figure 4. The DNA fragment was amplified by PCR using primers that incorporated useful restriction enzyme sites. A *Hind*III site was added just upstream of the TAA (stop codon) of the amylase coding region. This TAA is out of frame with the chicken amylase coding region, which brings in its own stop codon. The downstream end of the terminator is modified into an *Asc*I site.

EXAMPLE 5: *Drosophila melanogaster* α -amylase cDNA

Plaque lifts of a lambda gt10 library containing cDNAs from RNA of a *Drosophila melanogaster* Oregon-R strain were hybridized to a mouse salivary α -amylase cDNA probe (the 1650 bp *Pst*I restriction fragment of pMSa104; Hagenbuechle *et al.*, 1980, Cell 21: 179-187), under conditions of reduced stringency - 38°C in a hybridization buffer containing 50% formamide. Following hybridization, filters were washed in 1 X SSC, 0.1% SDS at 52°C.

Eight positive plaques were identified from an estimated 20,000 plaques screened. The longest of these cDNA inserts (OR-M7) was subcloned into a plasmid vector (pUC13) and subjected to DNA sequencing by standard methods (Benkel *et al.*, Genome 29: 510-515). The DNA sequence was determined as described in Boer & Hickey, 1986, Nucl. Acids Res. 14: 8399-8411). The extension products were analyzed on an Applied Biosystems 373A automated sequencer, and sequence assembly was performed using MicroGenie software by Beckman. OR-M7 was found to contain the complete coding region of the *Drosophila melanogaster* α -amylase pre-enzyme.

The DNA fragment encoding the mature *Drosophila melanogaster* α -amylase enzyme was inserted into expression vectors by PCR amplification using the LA-PCR kit and synthetic oligonucleotide primers. The oligonucleotide primers used for the PCR amplification were as follows: (i) *Dm-Q1* (5'-ATGCTAGCGCAATTCGACAC

CAACTACGCA-3', SEQID No: 6) which spans the position in the coding region corresponding to the N-terminus of the mature enzyme; and *Dm3-2* (5'-CAAGCTTGTTT ACAACTTGGCGTTGACGT-3', SEQID No. 7) which spans the stop codon of the amylase coding region. The primers were designed to amplify the region in the gene encoding the mature α -amylase peptide. In addition, *Dm-Q1* converts the environment of the signal peptidase cleavage site into an *NheI* site, while *Dm3-2* adds a *HindIII* site immediately downstream of the stop codon (enzyme recognition sites are underlined). The modifications introduced by the PCR primers allow the amplified cDNA to be inserted into the reporter construct using cohesive overhang ligation. The modified *Drosophila* α -amylase cDNA is SEQID No. 8.

EXAMPLE 6: The α -amylase dual-reporter gene system

Construction of the "test" and "control" plasmids began with the plasmid pIBI-B, which was formed from the vector pIBI25 (International Biotechnologies Inc.) by techniques of PCR amplification using primers with redundant overhangs (Scharf, 1990), and standard oligonucleotide linker addition (Sambrook et al., 1989). pIBI-B consists of: (i) *Drosophila* α -amylase promoter; (ii) the *Drosophila* α -amylase signal sequence, as described in Example 2; and (iii) the *Drosophila* α -amylase termination region, as described in Example 4.

20

The region spanning the chicken α -amylase cDNA was amplified by long-range PCR (LA-PCR kit, TaKaRa), incorporating a *NheI* site at the 5' primer and a *HindIII* site at the 3' primer. This PCR product was ligated into the plasmid pIBI-B via the *NheI* and *HindIII* sites to form the plasmid pIBI-fAmy, which contains chicken α -amylase joined to *Drosophila* amylase signal sequence. The CMV promoter was amplified by PCR from the plasmid pLNCX (Miller, D.M., & Rosman, G.J., 1989, BioTechniques, 7(9): 980-990), then inserted into the plasmid pIBI-fAmy, just upstream of the 5' end of the *Drosophila* signal sequence, replacing the *Drosophila* α -amylase promoter and forming the plasmid pIBI-CfAmy. The entire region spanning the CMV promoter to the 3' end of the chicken α -amylase gene was amplified by long-range PCR, incorporating unique sites at the 3' and 5' ends. This PCR

30

product was ligated to the 3110bp *HindIII-FseI* fragment of the plasmid pSEAP2-Basic (Clontech), forming the plasmid pUC-CFAmyHF. The *Drosophila* α -amylase gene, including the signal sequence, was amplified by PCR from the cDNA clone; the PCR product was inserted upstream of the chicken α -amylase cassette in the plasmid pUC-CFAmyHF to form the final plasmid pUC-TEST (SEQID No: 9). This "test" plasmid, shown in Figure 10, has the chicken α -amylase gene driven by the CMV promoter. The *Drosophila* α -amylase gene is not under any promoter; instead, there is a polylinker sequence 5' of the *Drosophila* α -amylase gene that allows promoters of interest to be inserted. In SEQID No:9 the components are located in the following positions: *Drosophila* α -amylase signal sequence: 83-134; *Drosophila* α -amylase: 135-1575; *Drosophila* α -amylase terminator: 1588-2128; CMV promoter: 2129-2951; *Drosophila* α -amylase signal sequence: 2952-2999; chicken amylase: 3000-4505; SV40 late polyadenylation site: 4506-4754; pUC origin of replication: 5033-5676; β -lactamase gene: 6684-582; β -lactamase promoter region: 6754-6685; F1 origin: 6816-7272; synthetic polyadenylation site: 7462-7450; and pause site from human α 2-globin gene: 7464-7555.

To construct the plasmid pUC-CONTROL, the rat β -actin promoter was inserted between the transcription block and the 5' end of the *Drosophila* α -amylase gene in the plasmid pUC-TEST. This plasmid (SEQID No: 10), shown schematically in Figure 10, has the chicken α -amylase gene driven by the CMV promoter, and the *Drosophila* α -amylase gene driven by the β -actin promoter. In SEQID No:10 the components are located in the following positions: pUC origin of replication: 5384-6027; β -lactamase gene: 7035-6175; β -lactamase promoter: 7105-7036; F1 origin: 7167-7623; synthetic polyadenylation site: 7753-7802; and pause site (human α 2-globin): 7815-7906.

EXAMPLE 7: The Chicken α -amylase reporter gene system

A variety of methods are available for the delivery of the reporter gene to the target host cells including: 1) liposome-mediated fusion; 2) micro-injection; 3) particle bombardment; 4) viral vectors; and 5) calcium phosphate co-precipitation.

For the purpose of this non-limiting illustrative example, the reporter gene linked to the CMV promoter has been inserted into the plasmid pIBI25 (International Biotechnologies Incorporated) to produce a transient expression construct. In addition, the reporter construct has been delivered to the cells using the calcium phosphate co-precipitation method. Other methods of DNA delivery are applicable, as is the use of vectors that promote the stable maintenance of the reporter gene within the transformed cells, e.g. retroviral vectors.

EXAMPLE 8: Harvesting of the Reporter Activity from Serum-Free Culture Supernatants:

Cells were grown under standard mammalian cell culture conditions. The media used to culture the cell lines were as follows: (i) for MA104 and Hela, Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal Bovine Serum; (ii) for CHO, DMEM with 10% Newborn Calf Serum; (iii) for PA317 and PG13, DMEM with iron-enriched 10% Calf Serum. Cells were plated at a density of 5×10^5 cells per 10 cm dish. Calcium phosphate transfection was carried out overnight. Following transfection, cells were incubated overnight in cell line-specific media as described above. On day 4, the culture medium was removed and replaced with Serum-free DMEM for all cell lines. On day 5, samples of medium were collected, and debris was removed from the samples by centrifugation at 500xg.

Cell supernatant was adjusted to 40% (vol/vol) of ethanol and the mixture incubated on ice for 1 hour. The solution was centrifuged at 16,000xg for 20 minutes at 4°C and the supernatant dried under vacuum to remove the ethanol. 100-150 µL of the resulting solution was used for routine analysis by native gel electrophoresis. To concentrate the solution, ~1mg of carrier protein (e.g. BSA) was added to the sample and the solution applied to a Centricon-30 filtration columns (Amicon). As little as 0.0005U of amylase processed in this manner can be detected by native gel electrophoresis.

EXAMPLE 9: Harvesting of the Reporter Activity from Serum-Containing Culture Supernatants:

Cells were grown under standard mammalian cell culture conditions as described above, except that 10% serum was included in the culture media. The procedure used to harvest the reporter protein is the same as that described for harvesting from milk (see below). The presence of serum gives some non-specific activity; however, this activity is clearly distinguishable from the reporter activity when detected by native gel electrophoresis (described in Example 12) because it has a different mobility. For an absolutely clean background and for concentrating the sample, adsorption on β -cyclodextrin (described in Example 11) can be used.

EXAMPLE 10: Harvesting of the Reporter Activity from Milk:

Two alternative methods are used to recover the α -amylase activity from milk. In the first method, fresh milk containing α -amylase was de-fatted by centrifugation at 8000xg, 10 min. at room temperature. The aqueous fraction was acid-curdled with 1M HCl to a final pH of 4.5, then centrifuged at 8000xg for 20 min. The whey (supernatant) fraction was neutralized with 1M NaOH to a final pH of 6.7. A portion of the final solution was used for analysis.

In the second method, the de-fatted milk was adjusted to 40% ethanol, placed on ice for 1-2h, then centrifuged at 16000xg at 4°C. The supernatant was then placed under vacuum to remove the ethanol, and a portion of the final solution was used for analysis with essentially complete recovery of the reporter activity. For concentrating the sample, adsorption on β -cyclodextrin without removing the ethanol (described in Example 11) can be used.

EXAMPLE 11: Harvesting of the Reporter Activity from Pre-lactation Secretion (Pre-milk):

Bovine pre-milk is a jelly-like substance that is so viscous it is nearly solid. To analyze α -amylase activity in the pre-milk, the sample was diluted 1/3-1/4 with water, then adjusted to 40% ethanol. The sample was placed on ice for 1-2h, then centrifuged

at 16000xg at 4°C to remove the precipitated proteins. β -cyclodextrin-Sepharose 6B, prepared as described in Vretblad, P., 1974, FEBS Lett. 47(1): 86-89, was added to the sample at a ratio of 40 μ L packed beads to 4mL of prepared sample. The sample was shaken for 2h at room temperature, then washed 5 times in 1.5mL of wash buffer (50mM Tris-HCl, 1mM CaCl₂ pH7.5). The sample was eluted by incubation for 5 min at room temperature in a desired volume of 10mg/mL β -cyclodextrin. As little as 0.0002U of α -amylase activity per mL of pre-milk can be recovered this way.

EXAMPLE 12: Detection of Reporter Activity

Amylase activity can be detected and measured by a variety of simple, safe procedures. These can be divided into the tube or liquid assays and the assays that involve diffusion in a semi-solid medium or electrophoretic separation.

The preferred protocol is electrophoretic separation of the proteins in the sample on a native (non-denaturing) polyacrylamide gel ("native gel electrophoresis"). This protocol is described in Benkel and Hickey (1986, Genetics 114: 137-144). Briefly, a low percentage acrylamide gel allows proteins to be separated on the basis of the overall charge of the molecules. Thus, even though chicken and mammalian amylases are very similar in molecular weight, they display very different migration patterns in the native gel assay (see Figure 5).

Following electrophoretic separation, the gel is incubated in a buffer solution containing partially-hydrolyzed starch. The starch granules coat the gel and penetrate the gel surface. Staining of the starch-coated gel with iodine results in a gel that shows clear amylase bands on a dark blue background.

The main advantage of the gel assay is that the background activity measured for the recipient cells is essentially zero. The gel assay is highly sensitive, and can easily be converted into a quantitative format by the incorporation of serial dilutions of an activity standard (see Benkel and Hickey, 1986, Genetics 114: 943-954). In

addition, there is a dye-linked starch-based substrate that facilitates real-time activity visualization.

As an alternative to native gel electrophoresis, prepared samples containing α -amylase can also be assayed by a very simple "plate diffusion" method. 1% agar, made in 50mM Tris-HCl, 1mM CaCl₂ pH 7.5, containing 0.02% starch, was poured onto culture plates. 4mm-diameter holes were made in the agar, into which up to 30 μ L of sample can be placed. The plates were covered and incubated overnight at 37°C to allow the amylase to diffuse. "Haloes", formed as the starch becomes digested, were detected by staining with iodine as described in the native gel electrophoresis procedure.

Chicken amylase in cell culture medium and in milk was tested and the activity was found to be stable for days at room temperature in a bioactive format. In addition, freezing and thawing does not affect the activity of the enzyme.

Figure 6 shows a variety of mammalian cell lines transformed with the reporter construct containing the CMV promoter delivered using a transient-expression vector. All cell lines tested including; CHO (hamster), ET-2 (cow), Hela (human), MA104 (monkey), PA317 and PG13 (mouse) showed production and secretion of chicken amylase according to the native gel assay. Different cell lines appear to secrete chicken amylase at different efficiencies. This may reflect the ability of the cells to take up foreign DNA, or it may be a measure of the inherent capacity of the different cell types to secrete proteins.

Figure 7 shows the results of an experiment in which PA317 cells were co-transfected with two reporter constructs: one containing chicken α -amylase as a reporter; the other contains firefly luciferase as a well-known intracellular reporter. In each co-transfection, both reporters are driven by the same promoter, which is the murine retroviral LTR, the rat β -actin promoter, or the human CMV early immediate promoter (see EXAMPLE 3 above for details of the promoters). The supernatants of

transiently transfected cells were harvested and analyzed for reporter activity as described in Example 8, while cell pellets were processed and analyzed for luciferase activity according to the instructions supplied by the luciferase assay kit manufacturer (Promega Corp). The results for extracellular amylase reporter activities show that the expression level of the CMV promoter is roughly four times that of the β -actin promoter, which in turn is about twice that of the LTR. Similar results were obtained with the intracellular luciferase promoter (Figure 12).

One of the primary applications for secreted reporter genes is in the optimization of DNA delivery and transformation *in vivo* of mammalian cells that secrete proteins in to biological fluids. This optimization process is crucial to the development of successful approaches for cell transformation in gene therapy. We are currently exploring the use of somatic transgenesis of bovine epithelial tissue in the development of 'bioreactor' cows for the production of pharmaceutical agents into milk. In order to compare the suitabilities of SEAP (Clontech Cat. No. K2041-1) and α -amylase as reporter gene/enzyme systems for the transformation of cow udder cells, SEAP and α -amylase were spiked into fresh cows' milk their activities measured. The SEAP used to spike the milk came from cell culture supernatant; the α -amylase was prepared from chicken pancreas.

20

Fresh cows' milk was centrifuged at 8,000 xg for 10 minutes at room temperature to remove the fat. SEAP cell supernatant and chicken α -amylase was added to the aqueous fraction. For the SEAP reconstitution experiment, SEAP activity was measured for the following treatments: (i) untreated milk aqueous phase ("Endogenous" in Table 1); (ii) milk aqueous phase heated to 65°C for 30 minutes ("Heated"); (iii) milk aqueous phase with SEAP spike ("Spiked"); and (iv) milk aqueous phase with SEAP spike heated to 65°C for 30 minutes ("Spiked/heated").

25

The results demonstrated that there is a high background of endogenous, soluble alkaline phosphatase activity in cows' milk. This activity is indistinguishable from SEAP. Heating the samples to 65°C does not decrease the endogenous SEAP activity

30

level; instead it appears that a portion of the endogenous alkaline phosphatase activity is present in an inactive, protein-complexed state in milk, and that heating tends to promote the release of some of this protein-bound endogenous SEAP activity. This results in a further increase in the background SEAP levels measured in the unspiked milk. The ability of milk proteins to inactivate SEAP is also evident from the dramatic decrease in SEAP signal measured in the spiked samples, where only about 1/4 of the SEAP activity added in the spike is detectable following the addition of the milk. Heating the spiked samples to 65°C raises the levels somewhat on average, but this increase in activity is at least partially due to the release of endogenous alkaline phosphatase enzyme.

TABLE 1: Measurement of SEAP activity in cow's milk.

Sample	Endogenous	Heated	Spiked	Spiked/heated
Cow 1	52	60	282	307
Cow 2	59	62	198	190

Legend: Cows' milk was processed as described in the text. SEAP activities are given in 1000's of luminometer units. "Endogenous" refers to the endogenous background activity present in the aqueous phase of the milk; "Heated" refers to the background activity in the milk following a 30 minute incubation at 65°C; "Spiked" refers to the unheated milk following the addition of the SEAP spike; "Spiked/heated" represents the spiked sample following a 30 minute incubation at 65°C. The SEAP spike consisted of 1,075 (x1000) units.

Increasing the level of the alkaline phosphatase-inhibitor homoarginine in the samples had no effect on the SEAP activity measurements. On the other hand, acid curdling of the milk to remove caseins, a standard method used to fractionate milk, resulted in the loss of the bulk of the SEAP spike. Likewise, adjusting the milk to 40% ethanol eliminates the SEAP spike, which precipitated out with the non-specific proteins.

Figure 8 shows milk samples with and without the addition of chicken α -amylase preparations. For whole milk, the gel assay shows a light smear throughout

the entire lane (see lane 1). This is due to the effect of the abundant milk casein proteins. However, the band of activity corresponding to the chicken amylase reporter 'spike' is clearly visible even in whole milk (lane 2). Our standard protocol for the amylase reconstitution experiments included an acid curdling step to remove caseins from the whole milk. This treatment was incompatible with the SEAP reporter system because it almost entirely depleted the milk aqueous phase of alkaline phosphatase activity (see above).

Following neutralization, the whey containing the chicken α -amylase spike was analyzed by the standard gel assay. This treatment completely eliminated any endogenous background activity from the milk samples, and left the chicken amylase band clearly visible in the spiked samples (lanes 7&8). Thus, chicken α -amylase is superior to SEAP as a secreted reporter enzyme in biological fluids such as cow's milk.

As outlined above, a major advantage of the amylase reporter system over existing reporter assays is the ability to detect chicken amylase activity in complex biological fluids. Cow's pre-lactation secretions (also known as pre-milk) are particularly problematic biological samples due to their extremely high content of proteins, fats and carbohydrates. However, using the simple β -cyclodextrin adsorption procedure described in Example 11, one can recover the chicken α -amylase reporter activity quantitatively even from such recalcitrant materials such as early and late pre-lactation secretions of pregnant heifers (see Figure 9). Note the complete lack of background activity for chicken amylase in the pre-milk of untreated udder quarters (lanes 1 & 6), and the presence of reporter activity only in udder quarters that were transfected with constructs encoding chicken α -amylase (lanes 2, 3, 4 & 5). In this way, as little as 0.0002U of α -amylase activity per mL of pre-milk can be detected.

Figure 10 gives a schematic representation of the dual reporter constructs used for promoter strength determinations in transiently transfected, culture cells. To measure the strength of a "test" promoter, the pUC-TEST plasmid is used. It consists

of two expression cassettes. The *Drosophila* α -amylase cassette has the *Drosophila* α -amylase cDNA, including the signal sequence and the terminator region. There is no promoter driving this reporter gene; instead, a polylinker region is provided immediately 3' of the cDNA to facilitate insertion of a promoter sequence of interest.

5 Just upstream of this polylinker region is a partially synthetic "transcriptional blocker" designed to minimize non-specific transcription of α -amylase. The *Drosophila* α -amylase gene is, therefore, the "test" isozyme. The second cassette has the chicken α -amylase cDNA joined at the 5' end with the signal sequence from *Drosophila melanogaster*; the terminator region for this cassette comes from the late polyadenylation site of SV40, to minimize possible recombination with the *Drosophila* α -amylase cassette. The chicken α -amylase gene is driven by the CMV promoter, and therefore, acts as the "normalizing" isozyme. The backbone of the plasmid pUC-based.

15 To provide a control showing that both cassettes are functional, the plasmid pUC-CONTROL is used. This plasmid is identical to pUC-TEST, except that the rat β -actin promoter was inserted upstream of the *Drosophila* α -amylase.

Figure 11 shows the results of transfecting PA317 cells in culture with the dual reporter construct. Lane 3 shows the lack of background activity in the culture medium of untransfected PA317 cells. Lane 4 contains culture medium from PA317 cells transfected with the plasmid pUC-TEST. The activity band is specific to the normalizing activity resulting from the expression of the chicken amylase reporter gene under the control of the CMV promoter. Lane 5 contains culture medium from PA317 cells transfected with the vector pUC-CONTROL. The chicken amylase band results from the expression of the normalizing isozyme while the *Drosophila melanogaster* (fly) amylase activity band results from the expression of the fly gene under the control of the β -actin promoter. The relative signal intensities of the two activity bands within the same gel provide a direct comparison of the transcriptional activities of the CMV and β -actin promoters. The β -actin promoter was inserted into pUC-CONTROL for the purposes of this example only. It will be obvious to persons skilled in the art that

the pUC-TEST assay system can be used to assay any nucleotide sequence whether natural or synthetic for promoter activity. Furthermore, by employing host cells other than PA317 cell line, the assay can be used to evaluate the effect of host cell type on promoter activity. In this particular instance, 80 μ L of culture supernatant, processed
5 as described in Example 8, was used.

All scientific publications and patent documents are incorporated herein by reference.

10 The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described in the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Her Majesty in Right of Canada as Rep. by
Agriculture and Agri-Food Canada
- (B) STREET: Central Experimental Farm
- (C) CITY: Ottawa
- (D) STATE: Ontario
- (E) COUNTRY: Canada
- (F) POSTAL CODE (ZIP): K1A 0C6

- (A) NAME: The University of Ottawa
- (B) STREET: 115 Seraphin Marion
- (C) CITY: Ottawa
- (D) STATE: Ontario
- (E) COUNTRY: Canada
- (F) POSTAL CODE (ZIP): K1N 6N5

(ii) TITLE OF INVENTION: Secreted alpha-Amylase as a Reporter Gene

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: CA 0,000,000

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: CM 2,203,613
- (B) FILING DATE: 24-APR-1997

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1505 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
GCTAGCTCAG TACAATCCCA ACACTCAGGC TGGGAGGACA TCTATCGTGC ATCTCTTTGA      60
ATGGCGCTGG GCCGACATTG CACTGGAGTG CGAACACTAT TTAGCTCCTA ATGGGTTTGG      120
```

AGGAGTTCAG	GTTTCTCCTC	CAAATGAAAA	CATTGTCATT	ACTAATCCGA	ACAGGCCCTG	180
GTGGGAAAGA	TACCAGCCCA	TCAGCTACAA	GATCTGCAGT	CGATCGGGCA	ATGAAAATGA	240
ATTCAGAGAC	ATGGTGACCA	GATGCAACAA	TGTTGGAGTT	CGTATTTATG	TGGATGCTGT	300
TGTCAATCAC	ATGTGTGGAT	CTATGGGTGG	CACGGGCACC	CACTCAACAT	GTGGGAGCTA	360
TTTCAACACC	GGGACTAGAG	ATTTTCCCGC	TGTGCCGTAC	TCTGCCTGGG	ATTTCAATGA	420
CGGCAAATGT	CACACTGCAA	GTGGAGACAT	CGAAAATTAT	GGGGACATGT	ATCAGGTCCG	480
GGATTGCAAG	TTGTCCAGCC	TTCTTGATCT	GGCTCTGGAG	AAGGACTATG	TACGCTCAAC	540
AATTGCAGCG	TACATGAATC	ACCTCATTGA	TATGGGTGTA	GCAGGGTTCC	GGATCGATGC	600
TGCCAAGCAT	ATGTGGCCAG	GGGACATAAG	AGCGTTTCTG	GACAAACTGC	ACGATCTAAA	660
TACTCAGTGG	TTTTCAGCAG	GAACGAAACC	CTTTATTTAC	CAAGAGGTAA	TTGACTTGGG	720
AGGAGAGCCA	ATCACAGGCA	GTCAGTACTT	TGGGAATGGC	CGCGTGACAG	AATTCAAGTA	780
TGGTGCCAAA	CTGGGGACGG	TGATCCGGAA	GTGGAATGGA	GAGAAGATGG	CCTACTTAAA	840
GAAGTGGGGA	GAAGGCTGGG	GCTTTGTGCC	TTCTGACAGA	GCCCTGGTGT	TTGTGGATAA	900
CCACGACAAC	CAGCGGGGGC	ACGGGGCAGG	CGGAGCTTCC	ATTCTTACTT	TCTGGGATGC	960
CAGGCTTTAT	AAAATGGCGG	TTGGTTTCAT	GCTCGCTCAT	CCGTACGGGT	TCACACGGGT	1020
GATGTCAAGT	TATCGTTGGC	CAAGATATTT	CGAAAACGGA	GTGGATGTTA	ACGACTGGGT	1080
GGGACCACCA	AGTAACTCGG	ACGGATCGAC	GAAGTCCGTT	ACAATCAACG	CAGACACTAC	1140
CTGTGGCAAT	GACTGGGTCT	GCGAACATCG	CTGGCGACAA	ATAAGGAACA	TGGTTATCTT	1200
CCGTAATGTG	GTAGACGGTC	AGCCTTTCTC	AAACTGGTGG	GACAACGGGA	GCAATCAAGT	1260
AGCTTTCGGT	CGCGGCGACA	GAGGCTTCAT	TGTCTTTAAT	AATGATGACT	GGTATATGAA	1320
TGTCGATTTG	CAAACTGGTC	TGCCTGCTGG	AACCTACTGC	GATGTTATTT	CTGGACAAAA	1380
GGAAGGCAGT	GCGTGACTG	GAAAGCAGGT	GTACGTTTCC	TCGGATGGAA	AGGCCAATTT	1440
CCAGATTAGT	AACAGCGATG	AAGATCCATT	TGTTGCAATT	CACGTTGATG	CCAAGTTATA	1500
AGCTT						1505

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 568 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AAGCTTGTAA ACAGCTGGGG AGCATGGCGA ACAGCCAGGC AATTAATTGA GATTATTAAT	60
TGTACGAAAT ATATATGATG AGATTATAAA CACACAACAC TTTTATTCGC AAGGGATGAT	120
AAGAATCTAA TATATATATT ATCTGGGCTT CAAAGCATTG ATTTTATTTA TTGAGTCAAG	180
AGGGAAATTT ATTTTCTTGT TATTCTCTGT CCAGGTCTAA AGTCCCGAGC GGTGAGGCTA	240
TCTATTGATT TGGACATTCC AATCGAATAC AAAACAGAGA TACAGAAATT TGCGAAAAAA	300
TTTGATAACA ATCGTGGATT TTACGAATTA GACAAATTGA TATGTGCTTG CTAATTGATG	360
TGGCATGAAA TAAGAAATTT ATAAGGACGT TTTCAAGTGC TTCTATTTTA AACATTGAGG	420
ATTTTTTTTT AAAGCAGACA GCTTTCAACA GGTTCGATGA GAATTTGAAT ATTGATTGTT	480
GACTTTAGCT ATACATAAAT CACACCTCAT CCACCCATTG TGGTATCCTT CGAAGGACTT	540
GGGAACTGGA TCCTCTAGAA GGC GCGCC	568

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATGCTAGCTC AGTACAATCC CAACACTCAG GCT	33
--------------------------------------	----

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

32

CGAAGCTTAT AACTTGGCAT CAACGTGAAT TG

32

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 56 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CCATGGTTCT GGCCAAGAGC ATAGTGTGCC TCGCCCTCCT GGCGGTGGCG CTAGCT

56

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATGCTAGCGC AATTGACAC CAACTACGCA

30

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CAAGCTTGTT TACAACTTGG CGTTGACGT

29

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1449 base pairs
 - (B) TYPE: nucleic acid

(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

ATGCTAGCGC AATTTCGACAC CAACTACGCA TCCGGTCGTA GTGGAATGGT CCACCTCTTC	60
GAGTGGAAGT GGGACGACAT CGCTGCCGAG TGCGAAAAC TCTTGGACC CAATGGCTAC	120
GCCGGTGTTT AGGTCTCCCC TGTGAACGAG AACGCCGTCA AGGACAGCCG CCCCTGGTGG	180
GAACGTTACC AGCCCATCTC CTACAAGCTG GAGACCCGCT CCGGAAACGA AGAGCAGTTC	240
GCCAGCATGG TCAAGCGCTG CAACGCCGTC GGAGTGCGCA CCTACGTGGA CGTGGTCTTC	300
AACCACATGG CCGCCGACGG AGGCACCTAC GGCCTGGCG GCAGCACCGC CAGCCCCAGC	360
AGCAAGAGCT ATCCCGGAGT GCCCTACTCC TCGCTGGACT TCAACCCGAC CTGCGCCATC	420
AGCAACTACA ACGACGCCAA CGAGGTGCGC AACTGCGAGC TGGTCGGTCT GCGCGACCTT	480
AACCAGGGCA ACTCTACGT GCAGGACAAG GTGGTCGAGT TCCTGGACCA TCTGATTGAT	540
CTCGGCGTGG CCGGATTCCG CGTGGACGCC GCCAAGCACA TGTGGCCCGC CGACCTGGCC	600
GTCATCTATG GCCGCCTCAA GAACCTAAAC ACCGACCACG GCTTCGCCTC GGGATCCAAG	660
GCGTACATCG TCCAGGAGGT CATCGACATG GCGGCGGAGG CCATCAGCAA GTCCGAGTAC	720
ACCGGACTGG GCGCCATCAC CGAGTTCCGC CACTCCGACT CCATCGGCAA GGTCTTCCGC	780
GGCAAGGACC AGTGCGAGTA CCTGACCAAC TGGGGCACC GCTGGGGCTT CGCTGCCTCC	840
GACCGCTCCC TGGTATTCGT CGACAACCAC GACAATCAGC GCGGACATGG AGCAGGAGGC	900
GCCGACGTTT TGACCTACAA GGTGCCCAAG CAGTACAAGA TGGCCTCCGC CTTTCATGCTG	960
GCGCACCCCT TCGGCACTCC CCGCGTGATG TCCTCCTTCT CCTTCACGGA CACCGATCAG	1020
GGCCCGCCCCA CCACCGACGG CCACAACATC GCCTCGCCCCA TCTTCAATAG CGACAACCTC	1080
TGCAGCGGCG GCTGGGTGTG TGAGCACCGC TGGCGCCAGA TCTACAACAT GGTGGCCTTC	1140
CGAAACACCG TGGGCTCGGA CGAGATCCAG AACTGGTGGG ACAACGGCAG CAACCAGATC	1200
TCCTTCAGCC GAGGCAGCCG CGGCTTCGTG GCCTTCAACA ACGACAACCTA CGACCTGAAC	1260
AGCTCCCTGC AGACGGGCCT GCCCGCCGGC ACCTACTGCG ACGTCATCTC CGGCTCCAAG	1320
AGCGGTTCCT CCTGCACGGG CAAGACCGTC ACCGTCGGAT CCGACGGACG GGCTTCCATC	1380

AACATTGGCA GCTCCGAGGA CGACGGAGTG CTGGCCATTC ACGTCAACGC CAAGTTGTAA 1440
 ACAAGCTTG 1449

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7562 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GGTACCGAGC TCTTACGCGT GCTAGCCCGG GCTCGAGCGG CCGCCAGTGT GATGGATATC 60
 TGCAGAATTC GGCTTAGTCG ACCCATGGTT CTGGCCAAGA GCATAGTGTG CCTCGECCTC 120
 CTGGCGGTGG CGCTAGCCCA ACGCCCAATT CGACACCAAC TACGCATCCG GTCGTAGTGG 180
 AATGGTCCAC CTCTTCGAGT GGAAGTGGGA CGACATCGCT GCCGAGTGCG AAAACTTCCT 240
 TGGACCCAAT GGCTACGCCG GTGTTCAGGT CTCCCCTGTG AACGAGAACG CCGTCAAGGA 300
 CAGCCGCCCC TGGTGGGAAC GTTACCAGCC CATCTCCTAC AAGCTGGAGA CCCGCTCCGG 360
 AAACGAAGAG CAGTTCGCCA GCATGGTCAA GCGCTGCAAC GCCGTCGGAG TGCGCACCTA 420
 CGTGGACGTG GTCTTCAACC ACATGGCCGC CGACGGAGGC ACCTACGGCA CTGGCGGCAG 480
 CACCGCCAGC CCCAGCAGCA AGAGCTATCC CGGAGTGCCC TACTCCTCGC TGGACTTCAA 540
 CCCGACCTGC GCCATCAGCA ACTACAACGA CGCCAACGAG GTGCGCAACT GCGAGCTGGT 600
 CGGTCTGCGC GACCTTAACC AGGGCAACTC CTACGTGCAG GACAAGGTGG TCGAGTTCCT 660
 GGACCATCTG ATTGATCTCG GCGTGGCCGG ATTCCGCGTG GACGCCGCCA AGCACATGTG 720
 GCCCCCGGAC CTGGCCGTCA TCTATGGCCG CCTCAAGAAC CTAAACACCG ACCACGGCTT 780
 CGCCTCGGGA TCCAAGGCGT ACATCGTCCA GGAGGTCATC GACATGGGCG GCGAGGCCAT 840
 CAGCAAGTCC GAGTACACCG GACTGGGCGC CATCACCGAG TTCCGCCACT CCGACTCCAT 900
 CGGCAAGGTC TTCCGCGGCA AGGACCAGCT GCAGTACCTG ACCAACTGGG GCACCGCCTG 960
 GGGCTTCGCT GCCTCCGACC GCTCCCTGGT ATTGTCGAC AACCACGACA ATCAGCGCGG 1020
 ACATGGAGCA GGAGGCGCCG ACGTTCGTGAC CTACAAGGTG CCAAGCAGT ACAAGATGGC 1080
 CTCCGCCTTC ATGCTGGCGC ACCCCTTCGG CACTCCCCGC GTGATGTCCT CTTCTCCTT 1140

CACGGACACC	GATCAGGGCC	CGCCCACCAC	CGACGGCCAC	AACATCGCCT	CGCCCATCTT	1200
CAATAGCGAC	AACTCCTGCA	GCGGCGGCTG	GGTGTGTGAG	CACCGCTGGC	GCCAGATCTA	1260
CAACATGGTG	GCCTTCCGAA	ACACCGTGGG	CTCGGACGAG	ATCCAGAACT	GGTGGGACAA	1320
CGGCAGCAAC	CAGATCTCCT	TCAGCCGAGG	CAGCCGCGGC	TTCGTGGCCT	TCAACAACGA	1380
CAACTACGAC	CTGAACAGCT	CCCTGCAGAC	GGGCTGCCC	GCCGGCACCT	ACTGCGACGT	1440
CATCTCCGGC	TCCAAGAGCG	GTTCTCCTG	CACGGGCAAG	ACCGTCACCG	TCGGATCCGA	1500
CGGACGGGCT	TCCATCAACA	TTGGCAGCTC	CGAGGACGAC	GGAGTGCTGG	CCATTCACGT	1560
CAACGCCAAG	TTGTAAGCTT	GTAAACAGCT	GGGGAGCATG	GCGAACAGCC	AGGCAATTAA	1620
TTGAGATTAT	TAATTGTACG	AAATATATAT	GATGAGATTA	TAAACACACA	ACACTTTTAT	1680
TCGCAAGGGA	TGATAAGAAT	CTAATATATA	TATTATCTGG	GCTTCAAAGC	ATTGATTTTA	1740
TTTATTGAGT	CAAGAGGGAA	ATTTATTTTC	TTGTTATTCT	CTGTCCAGGT	CTAAAGTCCC	1800
GAGCGGTGAG	GCTATCTATT	GATTTGGACA	TTCCAATCGA	ATACAAAACA	GAGATACAGA	1860
AATTTCCGAA	AAAATTTGAT	AACAATCGTG	GATTTTAGCA	ATTAGACAAA	TTGATATGTG	1920
CTTGCTAATT	GATGTGGCAT	GAAATAAGAA	ATTTATAAGG	ACGTTTTCAA	GTGCTTCTAT	1980
TTTAAACATT	CAGGATTTTT	TTTTAAAGCA	GACAGCTTTC	AACAGGTTTG	ATCAGAATTT	2040
GAATATTGAT	TGTTGACTTT	AGCTATACAT	AAATCACACC	TCATCCACCC	ATTGTGGTAT	2100
CCTTCGAAGG	ACTTGGGAAC	TGGATCCTCT	AGAGCTTCCG	GCCATTAGCC	ATATTATTCA	2160
TTGGTTATAT	AGCATAAATC	AATATTGGCT	ATTGGCCATT	GCATACGTTG	TATCCATATC	2220
ATAATATGTA	CATTTATATT	GGCTCATGTC	CAACATTACC	GCCATGTTGA	CATTGATTAT	2280
TGACTAGTTA	TTAATAGTAA	TCAATTACGG	GGTCATTAGT	TCATAGCCCA	TATATGGAGT	2340
TCCGCGTTAC	ATAACTTACG	GTAAATGGCC	CGCCTGGCTG	ACCGCCCAAC	GACCCCCGCC	2400
CATTGACGTC	AATAATGACG	TATGTTCCCA	TAGTAACGCC	AATAGGGACT	TTCCATTGAC	2460
GTCAATGGGT	GGAGTATTTA	CGGTAAACTG	CCCACTTGGC	AGTACATCAA	GTGTATCATA	2520
TGCCAAGTAC	GCCCCCTATT	GACGTCAATG	ACGGTAAATG	GCCCCGCTGG	CATTATGCCC	2580
AGTACATGAC	CTTATGGGAC	TTTCCTACTT	GGCAGTACAT	CTACGTATTA	GTCATCGCTA	2640
TTACCATGGT	GATGCGGTTT	TGGCAGTACA	TCAATGGGCG	TGGATAGCGG	TTTGACTCAC	2700
GGGGATTTCC	AAGTCTCCAC	CCCATTGACG	TCAATGGGAG	TTTGTTTTGG	CACCAAAATC	2760
AACGGGACTT	TCCAAAATGT	CGTAACAAC	CCGCCCCATT	GACGCAAATG	GGCGGTAGGC	2820

ATGTACGGTG	GGAGGTCTAT	ATAAGCAGAG	CTCGTTTAGT	GAACCGTCAG	ATCGCCTGGA	2880
GACGCCATCC	ACGCTGTTTT	GACCTCCATA	GAAGACACCG	GGACCGATCC	AGCCTCCGCG	2940
GCCCCAAGCT	CATGGTTCTG	GCCAAGAGCA	TAGTGTGCCT	CGCCCTCCTG	GCGGTGGCGC	3000
TAGCCAGTA	CAATCCCAAC	ACTCAGGCTG	GGAGGACATC	TATCGTGCAT	CTCTTTGAAT	3060
GGCGCTGGGC	CGACATTGCA	CTGGAGTGCG	AACACTATTT	AGCTCCTAAT	GGGTTTGGAG	3120
GAGTTCAGGT	TTCTCCTCCA	AATGAAAACA	TTGTCATTAC	TAATCCGAAC	AGGCCCTGGT	3180
GGGAAAGATA	CCAGCCCATC	AGCTACAAGA	TCTGCAGTCG	ATCGGGCAAT	GAAAATGAAT	3240
TCAGAGACAT	GGTGACCAGA	TGCAACAATG	TTGGAGTTTCG	TATTTATGTG	GATGCTGTTG	3300
TCAATCACAT	GTGTGGATCT	ATGGGTGGCA	CGGGCACCCA	CTCAACATGT	GGGAGCTATT	3360
TCAACACCGG	GACTAGAGAT	TTTCCCCTG	TGCCGTACTC	TGCCTGGGAT	TTCAATGACG	3420
GCAAATGTCA	CACTGCAAGT	GGAGACATCG	AAAATTATGG	GGACATGTAT	CAGGTCCGGG	3480
ATTGCAAGTT	GTCCAGCCTT	CTTGATCTGG	CTCTGGAGAA	GGACTATGTA	CGCTCAACAA	3540
TTGCAGCGTA	CATGAATCAC	CTCATTGATA	TGGGTGTAGC	AGGGTTCCGG	ATCGATGCTG	3600
CCAAGCATAT	GTGGCCAGGG	GACATAAGAG	CGTTTCTGGA	CAAACGCAC	GATCTAAATA	3660
CTCAGTGGTT	TTCAGCAGGA	ACGAAACCCT	TTATTTACCA	AGAGGTAATT	GACTTGGGAG	3720
GAGAGCCAAT	CACAGGCAGT	CAGTACTTTG	GGAATGGCCG	CGTGACAGAA	TTCAAGTATG	3780
GTGCCAAACT	GGGGACGGTG	ATCCGGAAGT	GGAATGGAGA	GAAGATGGCC	TACTTAAAGA	3840
ACTGGGGAGA	AGGCTGGGGC	TTGTGTCCTT	CTGACAGAGC	CCTGGTGTTT	GTGGATAACC	3900
ACGACAACCA	GCGGGGGCAC	GGGGCAGGCG	GAGCTTCCAT	TCTTACTTTC	TGGGATGCCA	3960
GGCTTTATAA	AATGGCGGTT	GGTTTCATGC	TCGCTCATCC	GTACGGGTTC	ACACGGGTGA	4020
TGTCAAGTTA	TCGTTGGCCA	AGATATTTTCG	AAAACGGAGT	GGATGTTAAC	GACTGGGTGG	4080
GACCACCAAG	TAATCGGAC	GGATCGACGA	AGTCCGTTAC	AATCAACGCA	GACACTACCT	4140
GTGGCAATGA	CTGGGTCTGC	GAACATCGCT	GGCGACAAAT	AAGGAACATG	GTTATCTTCC	4200
GTAATGTGGT	AGACGGTCAG	CCTTTCTCAA	ACTGGTGGGA	CAACGGGAGC	AATCAAGTAG	4260
CTTTCGGTCG	CGGCGACAGA	GGCTTCATTG	TCTTTAATAA	TGATGACTGG	TATATGAATG	4320
TCGATTTGCA	AAGTGGTCTG	CCTGCTGGAA	CCTACTGCGA	TGTTATTTCT	GGACAAAAGG	4380
AAGGCAGTGC	GTGTACTGGA	AAGCAGGTGT	ACGTTTCCTC	GGATGGAAAG	GCCAATTTCC	4440
AGATTAGTAA	CAGCGATGAA	GATCCATTTG	TTGCAATTCA	CGTTGATGCC	AAGTTATAAG	4500

GCCGGCCGCT	TCGAGCAGAC	ATGATAAGAT	ACATTGATGA	GTTTGGACAA	ACCACAATA	4560
GAATGCAGTG	AAAAAATGC	TTTATTTGTG	AAATTTGTGA	TGCTATTGCT	TTATTTGTAA	4620
CCATTATAAG	CTGCAATAAA	CAAGTTAACA	ACAACAATTG	CATTCATTTT	ATGTTTCAGG	4680
TTCAGGGGGA	GGTGTGGGAG	GTTTTTTAAA	GCAAGTAAAA	CCTCTACAAA	TGTGGTAAAA	4740
TCGATAAGGA	TCCGTCGACC	GATGCCCTTG	AGAGCCTTCA	ACCCAGTCAG	CTCCTTCCGG	4800
TGGGCGCGGG	GCATGACTAT	CGTCGCCGCA	CTTATGACTG	TCTTCTTTAT	CATGCAACTC	4860
GTAGGACAGG	TGCCGGCAGC	GCTCTTCCGC	TTCCTCGCTC	ACTGACTCGC	TGCGCTCGGT	4920
CGTTCGGCTG	CGGCGAGCGG	TATCAGCTCA	CTCAAAGGCG	GTAATACGGT	TATCCACAGA	4980
ATCAGGGGAT	AACGCAGGAA	AGAACATGTG	AGCAAAAGGC	CAGCAAAAGG	CCAGGAACCG	5040
TAAAAAGGCC	GCGTTGCTGG	CGTTTTTCCA	TAGGCTCCGC	CCCCCTGACG	AGCATCACAA	5100
AAATCGACGC	TCAAGTCAGA	GGTGGCGAAA	CCCGACAGGA	CTATAAAGAT	ACCAGGCGTT	5160
TCCCCCTGGA	AGCTCCCTCG	TGCGCTCTCC	TGTTCCGACC	CTGCCGCTTA	CCGGATACCT	5220
GTCCGCCTTT	CTCCCTTCGG	GAAGCGTGGC	GCTTTCTCAT	AGCTCACGCT	GTAGGTATCT	5280
CAGTTCGGTG	TAGGTCGTTT	GCTCCAAGCT	GGGCTGTGTG	CACGAACCCC	CCGTTTCAGCC	5340
CGACCGCTGC	GCCTTATCCG	GTAACATATG	TCTTGAGTCC	AACCCGGTAA	GACACGACTT	5400
ATCGCCACTG	GCAGCAGCCA	CTGGTAACAG	GATTAGCAGA	GCGAGGTATG	TAGGCGGTGC	5460
TACAGAGTTC	TTGAAGTGGT	GGCCTAATA	CGGCTACACT	AGAAGGACAG	TATTTGGTAT	5520
CTGCGCTCTG	CTGAAGCCAG	TTACCTTCGG	AAAAAGAGTT	GGTAGCTCTT	GATCCGGCAA	5580
ACAAACCACC	GCTGGTAGCG	GTGGTTTTTT	TGTTTGCAAG	CAGCAGATTA	CGCGCAGAAA	5640
AAAAGGATCT	CAAGAAGATC	CTTTGATCTT	TTCTACGGGG	TCTGACGCTC	AGTGGAACGA	5700
AAACTCACGT	TAAGGGATTT	TGGTCATGAG	ATTATCAAAA	AGGATCTTCA	CCTAGATCCT	5760
TTTAAATTAA	AAATGAAGTT	TTAAATCAAT	CTAAAGTATA	TATGAGTAAA	CTTGGTCTGA	5820
CAGTTACCAA	TGCTTAATCA	GTGAGGCACC	TATCTCAGCG	ATCTGTCTAT	TTGTTTCATC	5880
CATAGTTGCC	TGACTCCCCG	TCGTGTAGAT	AACTACGATA	CGGGAGGGCT	TACCATCTGG	5940
CCCCAGTGCT	GCAATGATAC	CGCGAGACCC	ACGCTCACC	GCTCCAGATT	TATCAGCAAT	6000
AAACCAGCCA	GCCGGAAGGG	CCGAGCGCAG	AAGTGGTCCT	GCAACTTTAT	CCGCCTCCAT	6060
CCAGTCTATT	AATTGTTGCC	GGGAAGCTAG	AGTAAGTAGT	TCGCCAGTTA	ATAGTTTGCG	6120
CAACGTTGTT	GCCATTGCTA	CAGGCATCGT	GGTGTACGCG	TCGTCGTTTG	GTATGGCTTC	6180

ATTCAGCTCC GGTTCCCAAC GATCAAGGCG AGTTACATGA TCCCCCATGT TGTGCAAAAA	6240
AGCGGTTAGC TCCTTCGGTC CTCCGATCGT TGTCAGAAGT AAGTTGGCCG CAGTGTATC	6300
ACTCATGGTT ATGGCAGCAC TGCATAATTC TCTTACTGTC ATGCCATCCG TAAGATGCTT	6360
TTCTGTGACT GGTGAGTACT CAACCAAGTC ATTCTGAGAA TAGTGTATGC GGCGACCGAG	6420
TTGCTCTTGC CCGGCGTCAA TACGGGATAA TACCGCGCCA CATAGCAGAA CTTTAAAAGT	6480
GCTCATCATT GGAAAACGTT CTTGCGGGCG AAAACTCTCA AGGATCTTAC CGCTGTTGAG	6540
ATCCAGTTCG ATGTAACCCA CTCGTGCACC CAACTGATCT TCAGCATCTT TTACTTTCAC	6600
CAGCGTTTCT GGGTGAGCAA AAACAGGAAG GCAAAATGCC GCAAAAAGG GAATAAGGGC	6660
GACACGGAAA TGTTGAATAC TCATACTCTT CCTTTTTCAA TATTATTGAA GCATTTATCA	6720
GGGTTATTGT CTCATGAGCG GATACATATT TGAATGTATT TAGAAAAATA AACAAATAGG	6780
GGTTCCGCGC ACATTTCCCC GAAAAGTGCC ACCTGACGCG CCCTGTAGCG GCGCATTAAAG	6840
CGCGGCGGGT GTGGTGGTTA CGCGCAGCGT GACCGCTACA CTTGCCAGCG CCCTAGCGCC	6900
CGCTCCTTTC GCTTTCTTCC CTTCTTTCT CGCCACGTTT GCCGGCTTTC CCCGTCAAGC	6960
TCTAAATCGG GGGCTCCCTT TAGGGTTCGG ATTTAGTGCT TTACGGCACC TCGACCCCAA	7020
AAAACTTGAT TAGGGTGATG GTTCACGTAG TGGGCCATCG CCCTGATAGA CGGTTTTTTCG	7080
CCCTTTGACG TTGGAGTCCA CGTCTTTTAA TAGTGGA CTC TTGTTCCAAA CTGGAACAAC	7140
ACTCAACCTT ATCTCGGTCT ATTCCTTTGA TTTATAAGGG ATTTTGCCGA TTTCGGCCTA	7200
TTGGTTAAAA AATGAGCTGA TTTAACAAAA ATTTAACGCG AATTTTAACA AAATATTAAC	7260
GTTTACAATT TCCCATTCGC CATTCAAGCT GCGCAACTGT TGGGAAGGGC GATCGGTGCG	7320
GGCCTCTTCG CTATTACGCC AGCCCAAGCT ACCATGATAA GTAAGTAATA TTAAGGTACG	7380
GGAGGTACTT GGAGCGGCCG CAATAAAATA TCTTTATTTT CATTACATCT GTGTGTTGGT	7440
TTTTTGTGTG AATCGATAGT ACTAACATAC GCTCTCCATC AAAACAAAAC GAAACAAAAC	7500
AAACTAGCAA AATAGGCTGT CCCAGTGCA AGTGCAGGTG CCAGAACATT TCTCTATCGA	7560
TA	7562

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7913 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGTACCGAAA CGCGCGAGGC AGCTGATCTG AGTCACTGAA TGGGATAGTG TCCACAAGGG	60
CGGGGGCTAT TCTTGTCAT CTGGGCCTAC GGAACCAGCA CCCATCGCCA AACTCTTCAT	120
CCTCTTCCTC AATCTCGCTT TCTCTCTCGT CCGCTTTTTT TTTCTTCTTC TTTTTTTTTT	180
TTTTTTTTTT TTTTTTTTGC AAAAGGAGGG GAGAGGGGGT AAAAAATGC TGCACGTGTC	240
GGCGAGGCCG GTGAGTGAGC GACGCGGAGC CAATCAGCGC CCGCCGTTCC GAAAGTTGCC	300
TTTTATGGCT CGAGTGGCCG CTGTGGCGTC CTATAAAACC CGGCGGCGCA ACGCGCAGCC	360
ACTGTCGAGT CAAGCTTCGA TCGTCGAGCG GCCGCCAGTG TGATGGATAT CTGCAGAATT	420
CGGCTTAGTC GACCCATGGT TCTGGCCAAG AGCATAGTGT GCCTCGCCCT CCTGGCGGTG	480
GCGCTAGCCC AACGCCAAT TCGACACCAA CTACGCATCC GGTGCTAGTG GAATGGTCCA	540
CCTCTTCGAG TGAAGTGGG ACGACATCGC TGCCGAGTGC GAAAACTTCC TTGGACCCAA	600
TGGCTACGCC GGTGTTCAAG TCTCCCCTGT GAACGAGAAC GCCGTCAAGG ACAGCCGCCC	660
CTGGTGGGAA CGTTACCAGC CCATCTCCTA CAAGCTGGAG ACCCGCTCCG GAAACGAAGA	720
GCAGTTCGCC AGCATGGTCA AGCGCTGCAA CGCCGTCGGA GTGCGCACCT ACGTGGACGT	780
GGTCTTCAAC CACATGGCCG CCGACGGAGG CACCTACGGC ACTGGCGGCA GCACCGCCAG	840
CCCCAGCAGC AAGAGCTATC CCGGAGTGCC CTACTCCTCG CTGGACTTCA ACCCGACCTG	900
CGCCATCAGC AACTACAACG ACGCCAACGA GGTGCGCAAC TCGAGCTGG TCGGTCTGCG	960
CGACCTTAAC CAGGGCAACT CCTACGTGCA GGACAAGGTG GTCGAGTTCC TGGACCATCT	1020
GATTGATCTC GGCGTGGCCG GATTCCGCGT GGACGCCGCC AAGCACATGT GGCCCGCCGA	1080
CCTGGCCGTC ATCTATGGCC GCCTCAAGAA CCTAAACACC GACCACGGCT TCGCCTCGGG	1140
ATCCAAGGCG TACATCGTCC AGGAGGTCAT CGACATGGGC GCGGAGGCCA TCAGCAAGTC	1200
CGAGTACACC GGAAGTGGCG CCATCACCAG GTTCCGCCAC TCCGACTCCA TCGCAAGGT	1260
CTTCCGCGGC AAGGACCAGC TGCAGTACCT GACCAACTGG GGCACCGCCT GGGGCTTCGC	1320
TGCCTCCGAC CGCTCCCTGG TATTGTCGA CAACCACGAC AATCAGCGCG GACATGGAGC	1380
AGGAGGCGCC GACGTTCTGA CCTACAAGGT GCCCAAGCAG TACAAGATGG CCTCCGCCTT	1440
CATGCTGGCG CACCCCTTCG GCACTCCCCG CGTGATGTCC TCCTTCTCCT TCACGGACAC	1500

CGATCAGGGC	CCGCCCACCA	CCGACGGCCA	CAACATCGCC	TCGCCCATCT	TCAATAGCGA	1560
CAACTCCTGC	AGCGGCGGCT	GGGTGTGTGA	GCACCGCTGG	CGCCAGATCT	ACAACATGGT	1620
GGCCTTCCGA	AACACCGTGG	GCTCGGACGA	GATCCAGAAC	TGGTGGGACA	ACGGCAGCAA	1680
CCAGATCTCC	TTCAGCCGAG	GCAGCCGCGG	CTTCGTGGCC	TTCAACAACG	ACAACCTACGA	1740
CCTGAACAGC	TCCCTGCAGA	CGGGCCTGCC	CGCCGGCACC	TACTGCGACG	TCATCTCCGG	1800
CTCCAAGAGC	GGTTCCTCCT	GCACGGGCAA	GACCGTCACC	GTCGGATCCG	ACGGACGGGC	1860
TTCCATCAAC	ATTGGCAGCT	CCGAGGACGA	CGGAGTGCTG	GCCATTCACG	TCAACGCCAA	1920
GTTGTAAGCT	TGTAAACAGC	TGGGGAGCAT	GGCGAACAGC	CAGGCAATTA	ATTGAGATTA	1980
TTAATTGTAC	GAAATATATA	TGATGAGATT	ATAAACACAC	AACACTTTTA	TTGCAAGGG	2040
ATGATAAGAA	TCTAATATAT	ATATTATCTG	GGCTTCAAAG	CATTGATTTT	ATTTATTGAG	2100
TCAAGAGGGA	AATTTATTTT	CTTGTTATTC	TCTGTCCAGG	TCTAAAGTCC	CGAGCGGTGA	2160
GGCTATCTAT	TGATTTGGAC	ATTCCAATCG	AATACAAAAC	AGAGATACAG	AAATTTCCGA	2220
AAAAATTTGA	TAACAATCGT	GGATTTTAGC	AATTAGACAA	ATTGATATGT	GCTTGCTAAT	2280
TGATGTGGCA	TGAAATAAGA	AATTTATAAG	GACGTTTTCA	AGTGCTTCTA	TTTTAAACAT	2340
TCAGGATTTT	TTTTTAAAGC	AGACAGCTTT	CAACAGGTTT	GATCAGAATT	TGAATATTGA	2400
TTGTTGACTT	TAGCTATACA	TAAATCACAC	CTCATCCACC	CATTGTGGTA	TCCTTCGAAG	2460
GACTTGGGAA	CTGGATCCTC	TAGAGCTTCC	GGCCATTAGC	CATATTATTC	ATTGGTTATA	2520
TAGCATAAAT	CAATATTGGC	TATTGGCCAT	TGCATACGTT	GTATCCATAT	CATAATATGT	2580
ACATTTATAT	TGGCTCATGT	CCAACATTAC	CGCCATGTTG	ACATTGATTA	TTGACTAGTT	2640
ATTAATAGTA	ATCAATTACG	GGGTCATTAG	TTCATAGCCC	ATATATGGAG	TTCCGCGTTA	2700
CATAACTTAC	GGTAAATGGC	CCGCCTGGCT	GACCGCCCAA	CGACCCCGC	CCATTGACGT	2760
CAATAATGAC	GTATGTTCCC	ATAGTAACGC	CAATAGGGAC	TTTCATTGA	CGTCAATGGG	2820
TGGAGTATTT	ACGGTAAACT	GCCCACTTGG	CAGTACATCA	AGTGTATCAT	ATGCCAAGTA	2880
CGCCCCCTAT	TGACGTCAAT	GACGGTAAAT	GGCCCGCCTG	GCATTATGCC	CAGTACATGA	2940
CCTTATGGGA	CTTTCCTACT	TGGCAGTACA	TCTACGTATT	AGTCATCGCT	ATTACCATGG	3000
TGATGCGGTT	TTGGCAGTAC	ATCAATGGGC	GTGGATAGCG	GTTTGACTCA	CGGGGATTTT	3060
CAAGTCTCCA	CCCCATTGAC	GTCAATGGGA	GTTTGTTTTG	GCACCAAAT	CAACGGGACT	3120
TTCCAAAATG	TCGTAACAAC	TCCGCCCCAT	TGACGCAAAT	GGGCGGTAGG	CATGTACGGT	3180

GGGAGGTCTA	TATAAGCAGA	GCTCGTTTAG	TGAACCGTCA	GATCGCCTGG	AGACGCCATC	3240
CACGCTGTTT	TGACCTCCAT	AGAAGACACC	GGGACCGATC	CAGCCTCCGC	GGCCCCAAGC	3300
TCATGGTTCT	GGCCAAGAGC	ATAGTGTGCC	TCGCCCTCCT	GGCGGTGGCG	CTAGCCCAGT	3360
ACAATCCCAA	CACTCAGGCT	GGGAGGACAT	CTATCGTGCA	TCTCTTTGAA	TGGCGCTGGG	3420
CCGACATTGC	ACTGGAGTGC	GAACACTATT	TAGCTCCTAA	TGGGTTTGA	GGAGTTCAGG	3480
TTTCTCCTCC	AAATGAAAAC	ATTGTCATTA	CTAATCCGAA	CAGGCCCTGG	TGGGAAAGAT	3540
ACCAGCCCAT	CAGCTACAAG	ATCTGCAGTC	GATCGGGCAA	TGAAAATGAA	TTCAGAGACA	3600
TGGTGACCAG	ATGCAACAAT	GTTGGAGTTC	GTATTTATGT	GGATGCTGTT	GTCAATCACA	3660
TGTGTGGATC	TATGGGTGGC	ACGGGCACCC	ACTCAACATG	TGGGAGCTAT	TTCAACACCG	3720
GGACTAGAGA	TTTTCCCGCT	GTGCCGTACT	CTGCCTGGGA	TTTCAATGAC	GGCAAATGTC	3780
AACTGCAAG	TGGAGACATC	GAAAATTATG	GGGACATGTA	TCAGGTCCGG	GATTGCAAGT	3840
TGTCCAGCCT	TCTTGATCTG	GCTCTGGAGA	AGGACTATGT	ACGCTCAACA	ATTGCAGCGT	3900
ACATGAATCA	CCTCATTGAT	ATGGGTGTAG	CAGGGTTCCG	GATCGATGCT	GCCAAGCATA	3960
TGTGGCCAGG	GGACATAAGA	GCGTTTCTGG	ACAAACTGCA	CGATCTAAAT	ACTCAGTGGT	4020
TTTCAGCAGG	AACGAAACCC	TTTATTTACC	AAGAGGTAAT	TGACTTGGGA	GGAGAGCCAA	4080
TCACAGGCAG	TCAGTACTTT	GGGAATGGCC	GCGTGACAGA	ATTCAAGTAT	GGTGCCAAAC	4140
TGGGGACGGT	GATCCGGAAG	TGGAATGGAG	AGAAGATGGC	CTACTTAAAG	AACTGGGGAG	4200
AAGGCTGGGG	CTTTGTGCCT	TCTGACAGAG	CCCTGGTGT	TGTGGATAAC	CACGACAACC	4260
AGCGGGGGCA	CGGGGCAGGC	GGAGCTTCCA	TTCTTACTTT	CTGGGATGCC	AGGCTTTATA	4320
AAATGGCGGT	TGGTTTCATG	CTCGCTCATC	CGTACGGGTT	CACACGGGTG	ATGTCAAGTT	4380
ATCGTTGGCC	AAGATATTTT	GAAAACGGAG	TGGATGTTAA	CGACTGGGTG	GGACCACCAA	4440
GTAACTCGGA	CGGATCGACG	AAGTCCGTTA	CAATCAACGC	AGACACTACC	TGTGGCAATG	4500
ACTGGGTCTG	CGAACATCGC	TGGCGACAAA	TAAGGAACAT	GGTTATCTTC	CGTAATGTGG	4560
TAGACGGTCA	GCCTTTCTCA	AACTGGTGGG	ACAACGGGAG	CAATCAAGTA	GCTTTTCGGTC	4620
GCGGCGACAG	AGGCTTCATT	GTCTTTAATA	ATGATGACTG	GTATATGAAT	GTCGATTGTC	4680
AAACTGGTCT	GCCTGCTGGA	ACCTACTGCG	ATGTTATTTT	TGGACAAAAG	GAAGGCAGTG	4740
CGTGTACTGG	AAAGCAGGTG	TACGTTTCCT	CGGATGGAAA	GGCCAATTTC	CAGATTAGTA	4800
ACAGCGATGA	AGATCCATTT	GTTGCAATTC	ACGTTGATGC	CAAGTTATAA	GGCCGGCCGC	4860

TTCGAGCAGA	CATGATAAGA	TACATTGATG	AGTTTGGACA	AACCACAAC	AGAATGCAGT	4920
GAAAAAATG	CTTTATTTGT	GAAATTTGTG	ATGCTATTGC	TTTATTTGTA	ACCATTATAA	4980
GCTGCAATAA	ACAAGTTAAC	AACAACAATT	GCATTCATTT	TATGTTTCAG	GTTCAGGGGG	5040
AGGTGTGGGA	GGTTTTTTAA	AGCAAGTAAA	ACCTCTACAA	ATGTGGTAAA	ATCGATAAGG	5100
ATCCGTCGAC	CGATGCCCTT	GAGAGCCTTC	AACCCAGTCA	GCTCCTTCCG	GTGGGCGCGG	5160
GGCATGACTA	TCGTCGCCGC	ACTTATGACT	GTCTTCTTTA	TCATGCAACT	CGTAGGACAG	5220
GTGCCGGCAG	CGCTCTTCCG	CTTCTCGCT	CACTGACTCG	CTGCGCTCGG	TCGTTCCGGT	5280
GCGGCGAGCG	GTATCAGCTC	ACTCAAAGGC	GGTAATACGG	TTATCCACAG	AATCAGGGGA	5340
TAACGCAGGA	AAGAACATGT	GAGCAAAAGG	CCAGCAAAAG	GCCAGGAACC	GTAAAAAGGC	5400
CGCGTTGCTG	GCGTTTTTCC	ATAGGCTCCG	CCCCCTGAC	GAGCATCACA	AAAATCGACG	5460
CTCAAGTCAG	AGGTGGCGAA	ACCCGACAGG	ACTATAAAGA	TACCAGGCGT	TTCCCCCTGG	5520
AAGCTCCCTC	GTGCGCTCTC	CTGTTCCGAC	CCTGCCGCTT	ACCGGATACC	TGTCCGCCTT	5580
TCTCCCTTCG	GGAAGCGTGG	CGCTTTCTCA	TAGCTCACGC	TGTAGGTATC	TCAGTTCGGT	5640
GTAGGTCGTT	CGCTCCAAGC	TGGGCTGTGT	GCACGAACCC	CCCGTTCAGC	CCGACCGCTG	5700
CGCCTTATCC	GGTAACTATC	GTCTTGAGTC	CAACCCGGTA	AGACACGACT	TATCGCCACT	5760
GGCAGCAGCC	ACTGGTAACA	GGATTAGCAG	AGCGAGGTAT	GTAGGCGGTG	CTACAGAGTT	5820
CTTGAAGTGG	TGGCCTAACT	ACGGCTACAC	TAGAAGGACA	GTATTTGGTA	TCTGCGCTCT	5880
GCTGAAGCCA	GTTACCTTCG	GAAAAAGAGT	TGGTAGCTCT	TGATCCGGCA	AACAAACCAC	5940
CGCTGGTAGC	GGTGGTTTTT	TTGTTTGCAA	GCAGCAGATT	ACGCGCAGAA	AAAAAGGATC	6000
TCAAGAAGAT	CCTTTGATCT	TTTCTACGGG	GTCTGACGCT	CAGTGGAACG	AAAACTCACG	6060
TTAAGGGATT	TTGGTCATGA	GATTATCAAA	AAGGATCTTC	ACCTAGATCC	TTTTAAATTA	6120
AAAATGAAGT	TTTAAATCAA	TCTAAAGTAT	ATATGAGTAA	ACTTGGTCTG	ACAGTTACCA	6180
ATGCTTAATC	AGTGAGGCAC	CTATCTCAGC	GATCTGTCTA	TTTCGTTTCAT	CCATAGTTGC	6240
CTGACTCCCC	GTCGTGTAGA	TAACCTACGAT	ACGGGAGGGC	TTACCATCTG	GCCCCAGTGC	6300
TGCAATGATA	CCGCGAGACC	CACGCTCACC	GGCTCCAGAT	TTATCAGCAA	TAAACCAGCC	6360
AGCCGGAAGG	GCCGAGCGCA	GAAGTGGTCC	TGCAACTTTA	TCCGCTCCA	TCCAGTCTAT	6420
TAATTGTTGC	CGGGAAGCTA	GAGTAAGTAG	TTCGCCAGTT	AATAGTTTGC	GCAACGTTGT	6480
TGCCATTGCT	ACAGGCATCG	TGGTGTACAG	CTCGTCGTTT	GGTATGGCTT	CATTCAGCTC	6540

CGGTTCCCAA	CGATCAAGGC	GAGTTACATG	ATCCCCCATG	TTGTGCAAAA	AAGCGGTTAG	6600
CTCCTTCGGT	CCTCCGATCG	TTGTCAGAAG	TAAGTTGGCC	GCAGTGTTAT	CACTCATGGT	6660
TATGGCAGCA	CTGCATAATT	CTCTTACTGT	CATGCCATCC	GTAAGATGCT	TTTCTGTGAC	6720
TGGTGAGTAC	TCAACCAAGT	CATTCTGAGA	ATAGTGTATG	CGGCGACCGA	GTTGCTCTTG	6780
CCCGGCGTCA	ATACGGGATA	ATACCGCGCC	ACATAGCAGA	ACTTTAAAAG	TGCTCATCAT	6840
TGGAAAACGT	TCTTCGGGGC	GAAAACCTCT	AAGGATCTTA	CCGCTGTTGA	GATCCAGTTC	6900
GATGTAACCC	ACTCGTGCAC	CCAACTGATC	TTCAGCATCT	TTTACTTTCA	CCAGCGTTTC	6960
TGGGTGAGCA	AAAACAGGAA	GGCAAAATGC	CGCAAAAAG	GGAATAAGGG	CGACACGGAA	7020
ATGTTGAATA	CTCATACTCT	TCCTTTTTCA	ATATTATTGA	AGCATTTATC	AGGGTTATTG	7080
TCTCATGAGC	GGATACATAT	TTGAATGTAT	TTAGAAAAAT	AAACAAATAG	GGGTTCCGCG	7140
CACATTTCCC	CGAAAAGTGC	CACCTGACGC	GCCCTGTAGC	GGCGCATTAA	GCGCGGCGGG	7200
TGTGGTGGTT	ACGCGCAGCG	TGACCGCTAC	ACTTGCCAGC	GCCCTAGCGC	CCGCTCCTTT	7260
CGCTTTCTTC	CCTTCCTTTC	TCGCCACGTT	CGCCGGCTTT	CCCCGTCAAG	CTCTAAATCG	7320
GGGGCTCCCT	TTAGGGTTCC	GATTTAGTGC	TTTACGGCAC	CTCGACCCCA	AAAAACTTGA	7380
TTAGGGTGAT	GGTTCACGTA	GTGGGCCATC	GCCCTGATAG	ACGGTTTTTC	GCCCTTTGAC	7440
GTTGGAGTCC	ACGTTCTTTA	ATAGTGGACT	CTTGTTCCAA	ACTGGAACAA	CACTCAACCC	7500
TATCTCGGTC	TATTCTTTTG	ATTTATAAGG	GATTTTGCCG	ATTTCGGCCT	ATTGGTTAAA	7560
AAATGAGCTG	ATTTAACAAA	AATTTAACGC	GAATTTTAAC	AAAATATTAA	CGTTTACAAT	7620
TTCCCATTCG	CCATTCAGGC	TGCGCAACTG	TTGGGAAGGG	CGATCGGTGC	GGGCCTCTTC	7680
GCTATTACGC	CAGCCCAAGC	TACCATGATA	AGTAAGTAAT	ATTAAGGTAC	GGGAGGTACT	7740
TGGAGCGGCC	GCAATAAAAAT	ATCTTTATTT	TCATTACATC	TGTGTGTTGG	TTTTTTGTGT	7800
GAATCGATAG	TACTAACATA	CGCTCTCCAT	CAAAACAAAA	CGAAACAAAA	CAAAC TAGCA	7860
AAATAGGCTG	TCCCCAGTGC	AAGTGCAGGT	GCCAGAACAT	TTCTCTATCG	ATA	7913

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An extra-cellular reporter gene system for use in a target cell, comprising a DNA sequence encoding:
 - a signal peptide;
 - at least one α -amylase protein; and
 - a transcription termination region.
2. The reporter gene system of claim 1, wherein the α -amylase protein is electrophoretically distinct from any endogenous amylase in the target cell and wherein the α -amylase protein is compatible with the target cells expression and secretion systems.
3. The reporter gene system of claim 2, wherein the reporter gene system comprises DNA sequences encoding two α -amylase proteins.
4. The reporter gene system of claim 3, wherein the α -amylase proteins are electrophoretically distinct from each other.
5. The reporter gene system of claim 2, wherein the α -amylase protein is selected from the group consisting of chicken α -amylase and *Drosophila melanogaster* α -amylase.
6. The reporter gene system of Claim 5, wherein the signal peptide is derived from a *D. melanogaster* α -amylase gene.
7. The reporter gene system of Claim 6, wherein the signal peptide is encoded by the DNA sequence of SEQID No: 5.

8. The reporter gene system of Claim 7, wherein the chicken α -amylase protein is encoded by the DNA sequence of SEQID No: 1.
9. The reporter gene system of Claim 7, wherein the *D. melanogaster* α -amylase protein is encoded by the DNA sequence of SEQID No: 8.
10. The reporter gene system of Claim 7, wherein the transcription termination region is derived from the group consisting of a *D. melanogaster* α -amylase gene and a late polyadenylation site of SV40.
11. The reporter gene system of Claim 10, wherein the transcription termination region is derived from the *D. melanogaster* α -amylase gene and is encoded by the DNA sequence of SEQID No: 2.
12. The reporter gene system of Claim 1, wherein said system further comprises a promoter region.
13. The reporter gene system of Claim 12, wherein the promoter region is selected from the group consisting of: a Cytomegalovirus promoter, a murine retroviral promoter and a rat β -actin promoter.
14. The reporter gene system of claim 12, wherein the α -amylase protein is electrophoretically distinct from any endogenous amylase in the target cell and wherein the α -amylase protein is compatible with the target cells expression and secretion systems.
15. The reporter gene system of claim 14, wherein the reporter gene system comprises DNA sequences encoding two α -amylase proteins.
16. The reporter gene system of claim 15, wherein the α -amylase proteins are electrophoretically distinct from each other.

17. The reporter gene system of claim 14, wherein the α -amylase protein is selected from the group consisting of chicken α -amylase and *Drosophila melanogaster* α -amylase.
18. The reporter gene system of Claim 17, wherein the signal peptide is derived from a *D. melanogaster* α -amylase gene.
19. The reporter gene system of Claim 18, wherein the signal peptide is encoded by the DNA sequence of SEQID No: 5.
20. The reporter gene system of Claim 17, wherein the chicken α -amylase protein is encoded by the DNA sequence of SEQID No: 1.
21. The reporter gene system of Claim 17, wherein the *D. melanogaster* α -amylase protein is encoded by the DNA sequence of SEQID No: 8.
22. The reporter gene system of Claim 17, wherein the transcription termination region is derived from the group consisting of a *D. melanogaster* α -amylase gene and a late polyadenylation site of SV40.
23. The reporter gene system of Claim 22, wherein the transcription termination region is derived from the *D. melanogaster* α -amylase gene and is encoded by the DNA sequence of SEQID No: 2.
24. The reporter gene system of Claim 11, wherein the extra-cellular reporter gene system comprising a DNA sequence encoding:
- a signal peptide derived from a *Drosophila melanogaster* α -amylase gene;
 - a chicken α -amylase protein; and
 - a transcription termination region derived from a *Drosophila melanogaster* α -amylase gene.

25. The reporter gene system of Claim 23, wherein the extra-cellular reporter gene system comprising a DNA sequence encoding:

- a Cytomegalovirus promoter,
- a signal peptide derived from a *Drosophila melanogaster* α -amylase gene;
- a chicken α -amylase protein; and
- a transcription termination region derived from a *Drosophila melanogaster* α -amylase gene.

26. The reporter gene system of Claim 11, wherein the reporter gene system comprising a DNA sequence encoding:

- a signal peptide derived from a *Drosophila melanogaster* α -amylase gene;
- a *Drosophila* α -amylase protein; and
- a transcription termination region derived from a *Drosophila melanogaster* α -amylase gene.

27. The reporter gene system of Claim 23, wherein the extra-cellular reporter gene system comprising a DNA sequence encoding:

- a Cytomegalovirus promoter,
- a signal peptide derived from a *Drosophila melanogaster* α -amylase gene;
- a *Drosophila* α -amylase protein; and
- a transcription termination region derived from a *Drosophila melanogaster* α -amylase gene.

28. The reporter gene system of Claim 16, wherein the extra-cellular reporter gene system comprising a DNA sequence encoding:

two expression cassettes, wherein the first cassette comprises a DNA sequence encoding:

- a signal peptide derived from a *Drosophila melanogaster* α -amylase gene;
- a *Drosophila* α -amylase protein; and
- a transcription termination region derived from a *Drosophila melanogaster* α -amylase gene;

and wherein said second cassette comprises a DNA sequence encoding:

- a Cytomegalovirus promoter,
- a signal peptide derived from a *D. melanogaster* α -amylase gene;
- a chicken α -amylase protein; and
- a transcription termination region derived from a late polyadenylation site of SV40;

wherein the first cassette further comprises a polylinker region immediately 3' of the *D. melanogaster* α -amylase gene to facilitate insertion of a promoter sequence of interest to determine the strength of said promoter.

29. The reporter gene system of any of the preceding claims where the target cell is a mammalian cell.

30. A kit comprising the reporter gene system of any of the preceding claims, a means of detecting the amylase activity and instructions for use.

31. The kit of claim 30 further comprising a beta-dextrin system to remove non-specific proteins from high-protein fluids.

32. The kit of claim 30 or 31, wherein the means of detecting the amylase system is selected from the group consisting of a plate assay and a gel electrophoresis means.

1/11

5'-GCTAGCTCAG TACAATCCCA ACACTCAGGC TGGGAGGACA TCTATCGTGC ATCTCTTTGA
ATGGCGCTGG GCCGACATTG CACTGGAGTG CGAACACTAT TTAGCTCCTA ATGGGTTTGG
AGGAGTTCAG GTTTCCTC CAAATGAAAA CATTGTCATT ACTAATCCGA ACAGGCCCTG
GTGGGAAAGA TACCAGCCCA TCAGCTACAA GATCTGCAGT CGATCGGGCA ATGAAAATGA
ATTCAGAGAC ATGGTGACCA GATGCAACAA TGTTGGAGTT CGTATTTATG TGGATGCTGT
TGTCATCAC ATGTGTGGAT CTATGGGTGG CACGGGCACC CACTCAACAT GTGGGAGCTA
TTTCAACACC GGGACTAGAG ATTTTCCCGC TGTGCCGTAC TCTGCCTGGG ATTTCAATGA
CGGCAAATGT CACACTGCAA GTGGAGACAT CGAAAATTAT GGGGACATGT ATCAGGTCCG
GGATTGCAAG TTGTCCAGCC TTCTTGATCT GGCTCTGGAG AAGGACTATG TACGCTCAAC
AATTGCAGCG TACATGAATC ACCTCATTGA TATGGGTGTA GCAGGGTTCC GGATCGATGC
TGCCAAGCAT ATGTGGCCAG GGGACATAAG AGCGTTTCTG GACAACTGC ACGATCTAAA
TACTCAGTGG TTTTCAGCAG GAACGAAACC CTTTATTTAC CAAGAGGTAA TTGACTTGGG
AGGAGAGCCA ATCACAGGCA GTCAGTACTT TGGGAATGGC CGCGTGACAG AATTCAAGTA
TGGTGCCAAA CTGGGGACGG TGATCCGGAA GTGGAATGGA GAGAAGATGG CCTACTTAAA
GAACTGGGGA GAAGGCTGGG GCTTTGTGCC TTCTGACAGA GCCCTGGTGT TTGTGGATAA
CCACGACAAC CAGCGGGGGC ACGGGGCAGG CGGAGCTTCC ATTCTTACTT TCTGGGATGC
CAGGCTTTAT AAAATGGCGG TTGGTTTCAT GCTCGCTCAT CCGTACGGGT TCACACGGGT
GATGTCAAGT TATCGTTGGC CAAGATATTT CGAAAACGGA GTGGATGTTA ACGACTGGGT
GGGACCACCA AGTAACTCGG ACGGATCGAC GAAGTCCGTT ACAATCAACG CAGACACTAC
CTGTGGCAAT GACTGGGTCT GCGAACATCG CTGGCGACAA ATAAGGAACA TGGTTATCTT
CCGTAATGTG GTAGACGGTC AGCCTTTCTC AAACCTGGTG GACAACGGGA GCAATCAAGT
AGCTTTCGGT CGCGGCGACA GAGGCTTCAT TGTCTTTAAT AATGATGACT GGTATATGAA
TGTCGATTG CAAACTGGTC TGCCTGCTGG AACCTACTGC GATGTTATTT CTGGACAAAA
GGAAGGCAGT GCGTGACTG GAAAGCAGGT GTACGTTTCC TCGGATGGAA AGGCCAATTT
CCAGATTAGT AACAGCGATG AAGATCCATT TGTTGCAATT CACGTTGATG CCAAGTTATA
AGCTT-3'

Figure 1

2/11

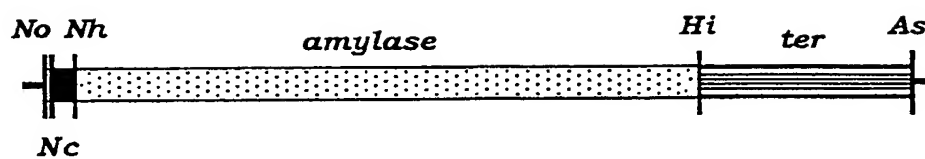


Figure 2

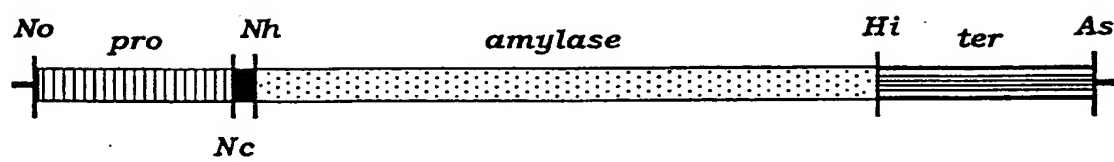


Figure 3

3/11

5'-AAGCTTGTAACACAGCTGGGGAGCATGGCGAACAGCCAGGC AATTAATTGA GATTATTAAT
TGTACGAAATATATATGATGAGATTATAAACACACAACAC TTTTATTCGC AAGGGATGAT
AAGAATCTAATATATATATTATCTGGGCTTCAAAGCATTGATTTTATTTA TTGAGTCAAG
AGGGAAATTTATTTTCTTGT TATTCTCTGTCCAGGTCTAAGTCCCGAGCGGTGAGGCTA
TCTATTGATT TGGACATTCC AATCGAATACAAAACAGAGATACAGAAATT TGCGAAAAAA
TTTGATAACAATCGTGGATT TTACGAATTAGACAAATTGATATGTGCTTGCTAATTGATG
TGGCATGAAATAAGAAATTTATAAGGACGT TTTCAAGTGTCTATTTTA AACATTCAGG
ATTTTTTTTTAAAGCAGACAGCTTTCAACAGGTTTGATGA GAATTTGAATATTGATTGTT
GACTTTAGCTATACATAAATCACACCTCATCCACCCATTG TGGTATCCTT CGAAGGACTT
GGGAAGTGA TCCTCTAGAA GCGCGGCC-3'

Figure 4

4/11

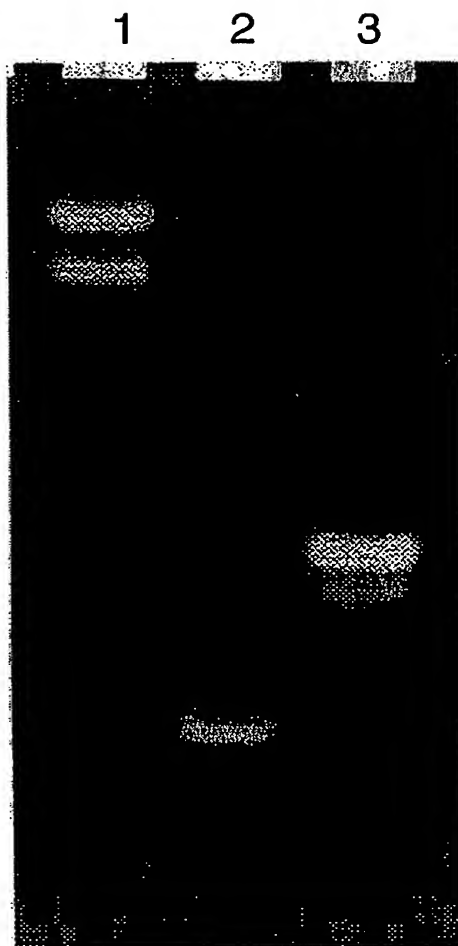


FIG. 5

5/11

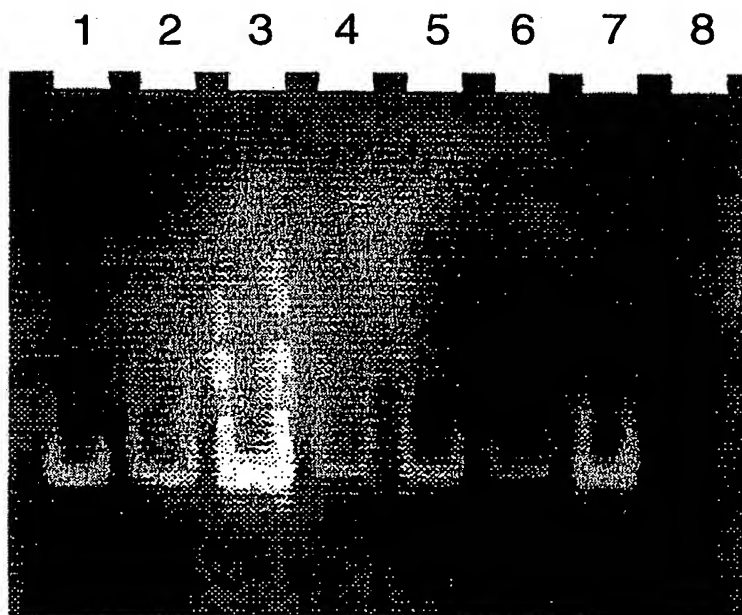


FIG. 6

6/11

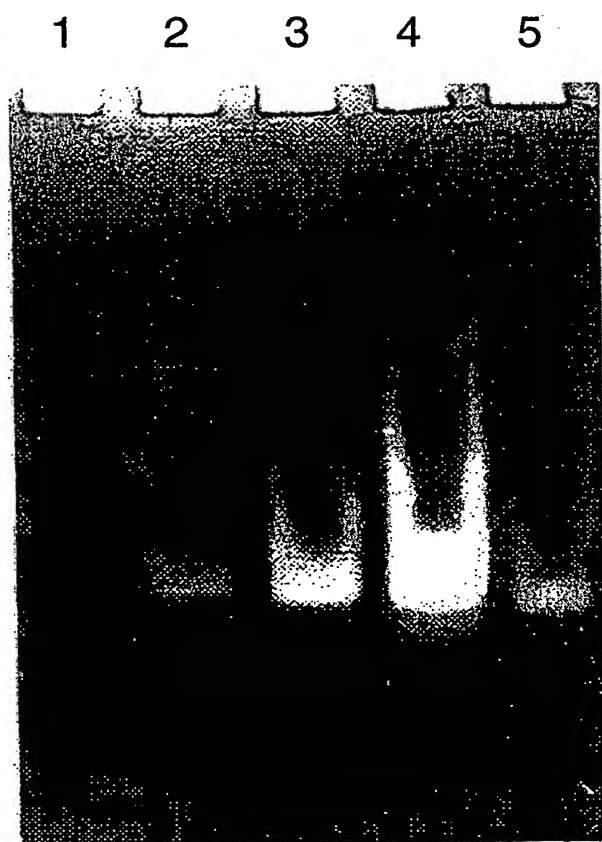


FIG. 7

7/11

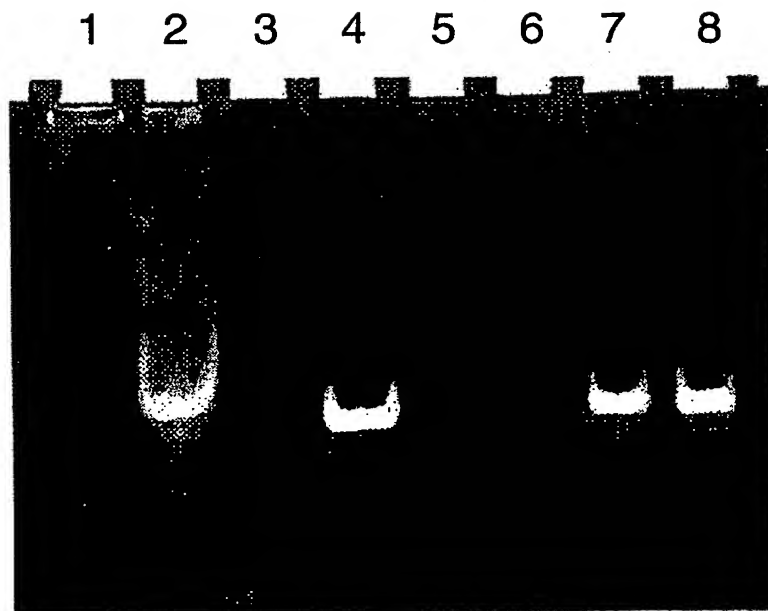


FIG. 8

8/11

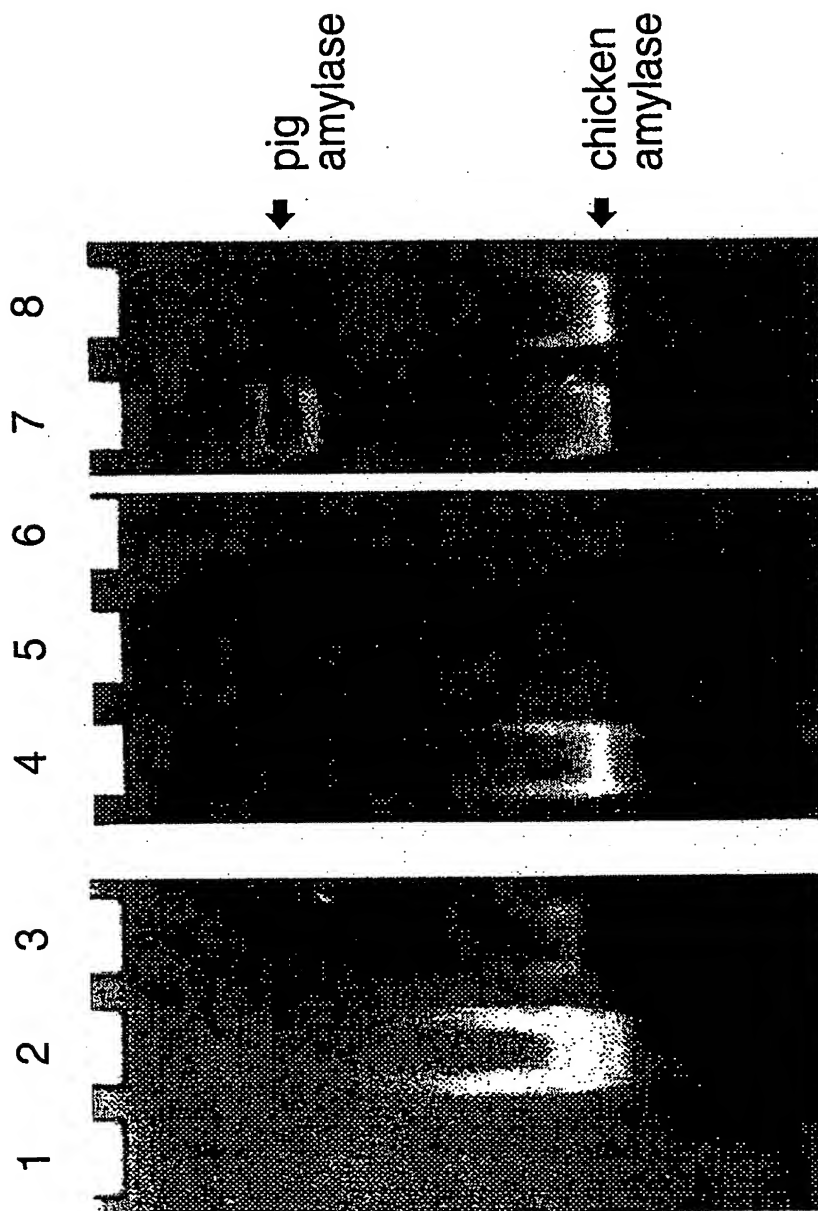


FIG. 9

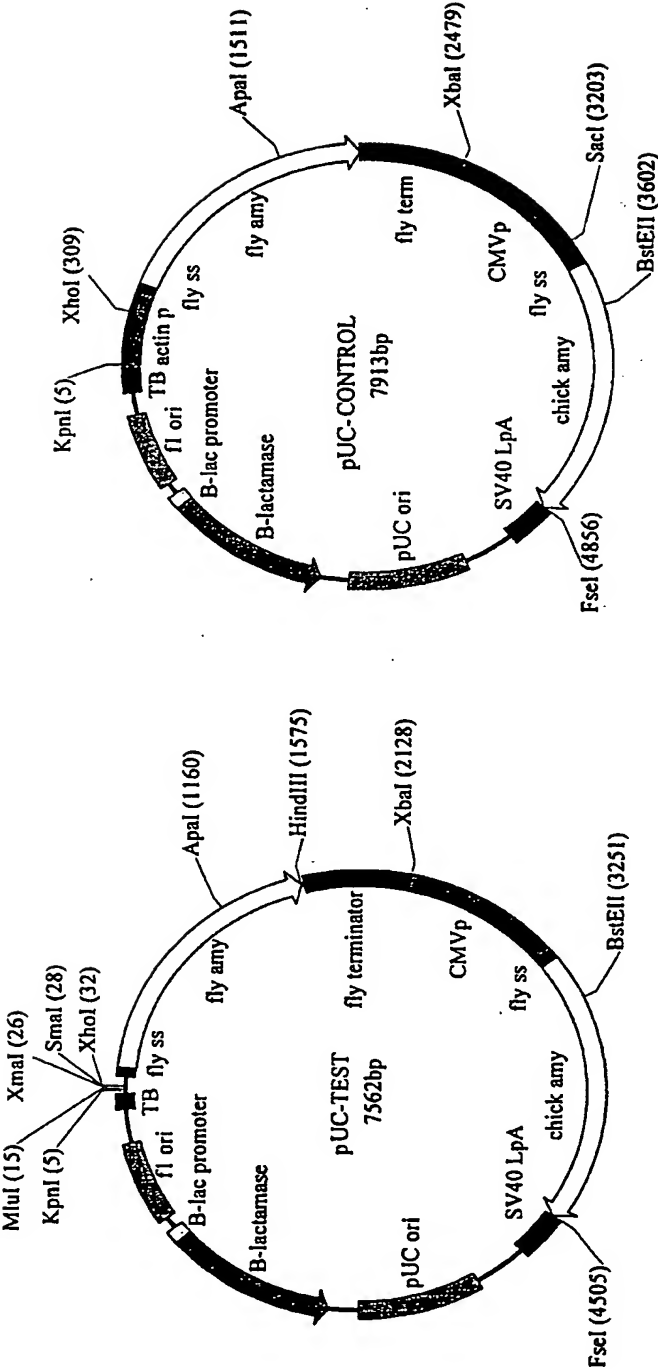


Figure 10

10/11

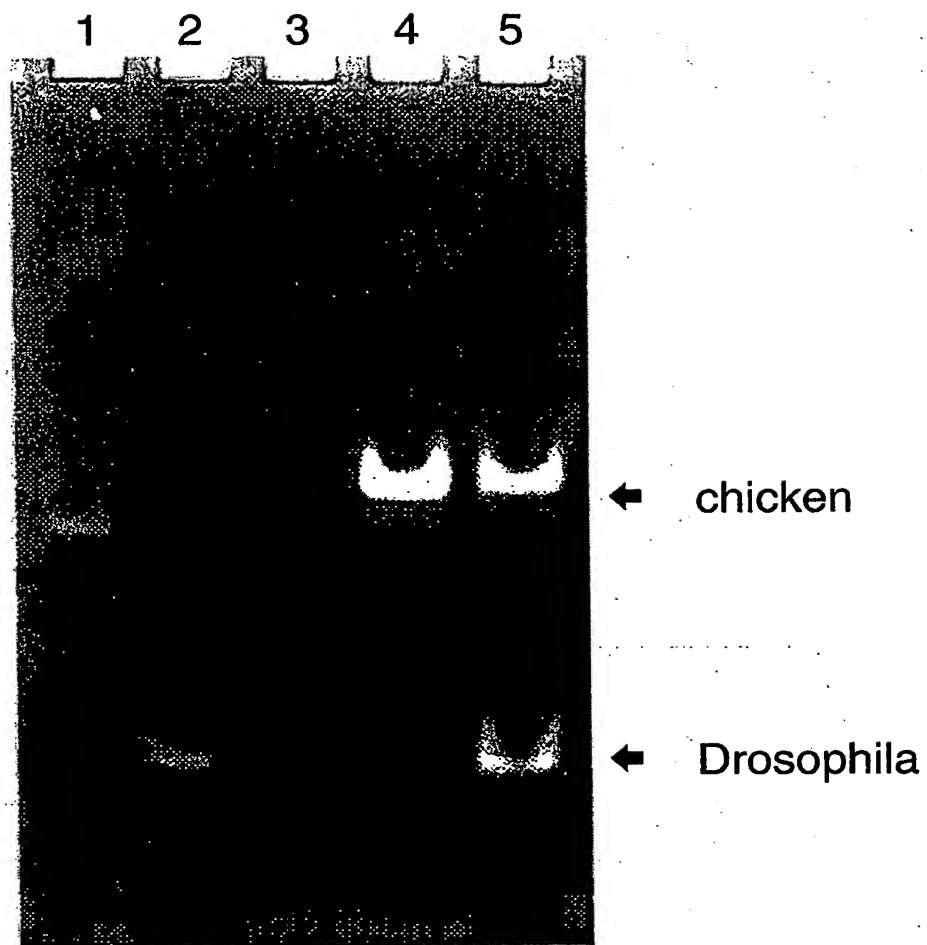


FIG. 11

11/11

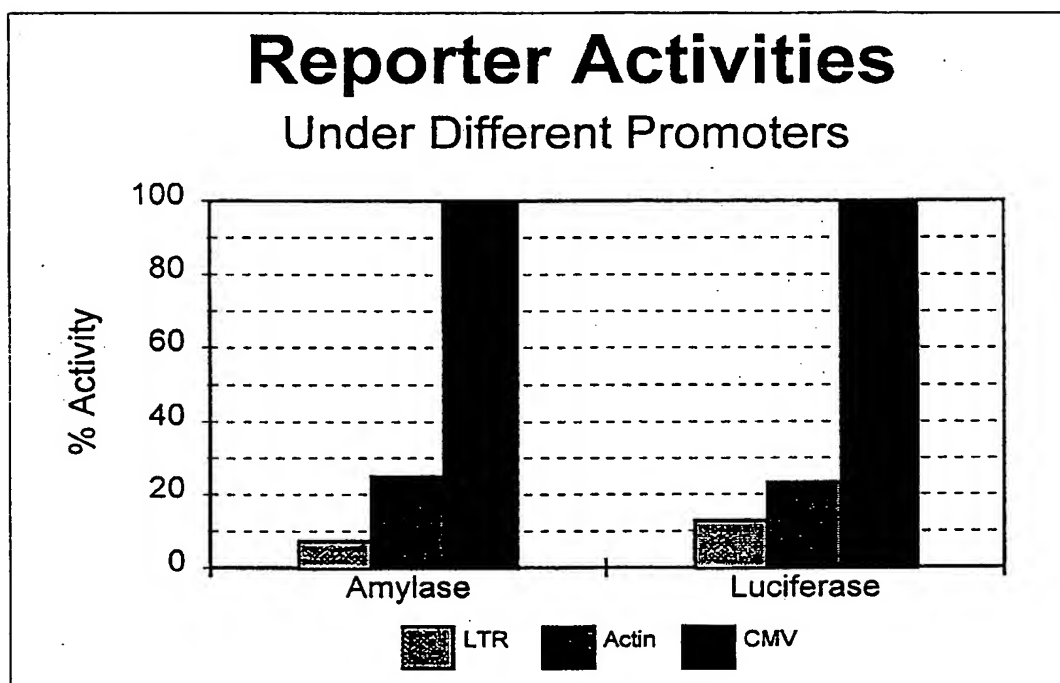


Figure 12

INTERNATIONAL SEARCH REPORT

National Application No

PCT/CA 98/00157

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/56 C12N15/85 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 438 200 A (CIGB) 24 July 1991 see the whole document ----	1, 2, 12, 30, 32
A	BOER, P.H. AND HICKEY, D.A.: "The alpha-amylase gene in Drosophila melanogaster: nucleotide sequence, gene structure and expression motifs" NUCLEIC ACIDS RESEARCH, vol. 14, no. 21, 1986, pages 8399-8411, XP002068035 see the whole document ----- -/-	1



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

15 June 1998

Date of mailing of the international search report

06/07/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Hillenbrand, G

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 98/00157

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>INOMATA, N. ET AL.: "Evolutionary relationships and sequence variation of alpha-amylase variants encoded by duplicated genes in the amy locus of Drosophila melanogaster" GENETICS, vol. 141, 1995, pages 237-244, XP002068036 see the whole document</p>	1
A	<p>WISE, R.J. ET AL.: "A complementary DNA sequence that predicts a human pancreatic amylase primary structure consistent with the electrophoretic mobility of the common isozyme, amy2 A." MOL. BIOL. MED., vol. 2, 1984, pages 307-322, XP002068037 see the whole document</p>	1

Information on patent family members

PCT/CA 98/00157

Form PCT/ISA/210 (patent family annex) (July 1992)